UNIVERSITY COLLEGE LONDON

DEVELOPMENT OF ADULT HEPATOCYTE CULTURE SYSTEMS CAPABLE OF EXPRESSING THE GENES ~~THAT CODE~~ CODING FOR THE MAJOR PROTEINS RESPONSIBLE FOR FOREIGN COMPOUND METABOLISM

A DISSERTATION SUBMITTED TO THE FACULTY OF LIFE SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

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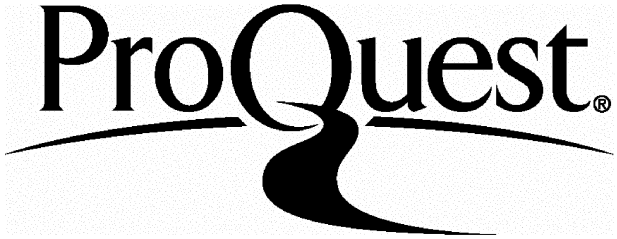

Abstract

Cytochromes P450 play a central role in the synthesis of endogenous compounds, such as steroids and fatty acids, and in the metabolism of many foreign compounds, including dietary components, therapeutic drugs and carcinogens. The expression of several cytochromes P450 is increased considerably upon exposure to certain xenobiotics. For example, treatment with the antiepileptic drug phenobarbital causes a dramatic increase in the abundance in rat liver of mRNAs encoding two members of the CYP2B subfamily, namely CYP2B1 and CYP2B2. Until recently, however, the phenobarbital response could not be reproduced in culture and therefore studies of the regulation of the expression of CYP2B1/2 have been hampered by the absence of a suitable cell culture system.

We have developed culture conditions for both primary rat hepatocytes and a highly differentiated rat hepatoma cell line, FAZA 967, that support the induction of *CYP2B* gene expression by phenobarbital. Using the RNase protection method we found that the induction of *CYP2B1/2* mRNAs is comparable in magnitude to that observed *in vivo*. We found also that in these cell systems, as is the case in rat liver, *CYP2B1/2* gene expression is induced by the barbiturate-antagonist picrotoxin, at levels comparable to those observed using phenobarbital as the inducer.

The expression and induction by phenobarbital of other drug-metabolising enzymes, namely P450 reductase, FMO1, GST1-1/2-2, 3-3/4-4, and GST7-7, has also been monitored using immunoblotting, and found to reflect the *in vivo* situation.

To demonstrate the possibility of using the primary hepatocyte system in promoter mapping studies, we have developed a highly efficient protocol for the transfection of DNA into these cells, based on lipofection. We demonstrated that in this system the immediate early promoter of human cytomegalovirus can promote the expression in large amounts of catalytically active β-galactosidase and luciferase enzymes. In initial attempts aimed at defining regions of the *CYP2B2* gene promoter important for its regulation, we found that a DNA fragment located between -368 and -4 of the 5'-flanking region of a *CYP2B2* gene was not sufficint to drive the expression of the *luciferase* gene.

Finally, we have attempted to establish a conditionally immortalised hepatocyte cell system by isolating hepatocytes from the livers of transgenic mice, harbouring a mutant, temperature sensitive form, of the SV40 virus large T antigen, whose expression is under the regulation of a promoter that is responsive to γ-interferon. These cells could be kept in culture for longer than a month, could be passaged and secreted albumin.

Questa tesi é dedicata ai miei genitori, a Natali ed alla memoria della cara Nonna Concetta

(this thesis is dedicated to my parents, to Natali, and to the memory of my dear Nonna Concetta)

Contents

List of figures and tables

Figures

Tables

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Questa tesi ed il lavoro in essa descritto non potevano essere realizzati senza l'aiuto di molte persone ed organizzazioni.

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ABBREVIATIONS

°C	degree Celsius
3-MC	3-methylcholanthrene
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
Ci	Curie
CLOF	clofibrate
cpm	counts per minute
CYP	cytochrome P450
Da	Daltons
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacidic acid
EGTA	ethylene glycol-*bis* (β-amino-ethyl ether) *N, N, N', N'* tetraacidic acid
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FMN	flavin mononucleotide
g	gram } as appropriate
g	unit of gravity } as appropriate
GSH	glutathione, reduced
GST	glutathione S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high-performance liquid chromatography

hr	hour(s)
i.p.	intra-peritoneal
i.v.	intravenous
Kb	kilobase
M	molar concentration
min	minute(s)
MOPS	3-[N-morpholino] propane sulphonic acid
mRNA	messenger RNA
n	nano- (x 10^{-9}) } as appropriate
n	number in a study or group
NAD(P)H	nicotinamide adenine (phosphate) dinucleotide reduced
nt	nucleotide
P	probability
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbons
PB	phenobarbital
PBS	phosphate-buffered saline
PCX	picrotoxin
pH	hydrogen ion exponent
Ph	phenyl ring
PIPES	[1,4-piperazine*bis*(ethane sulphonic acid)
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SV40	Simian Virus 40
Tris	N-tris(hydroxymethyl)aminomethane

tRNA	transfer RNA
UTP	uridine triphosphate
UV	ultraviolet
V	Volt
v	volume
w	weight

Chapter one

Introduction

1.1 Pathways of drug metabolism

All living organisms are continually exposed to an incredibly wide array of foreign chemical compounds (xenobiotics) that have the potential to interact with cellular macromolecules (DNA, RNA, and proteins) and thus cause various cytotoxic and genotoxic effects. This has pressured organisms (from bacteria to plants to humans) to evolve complex systems of detoxification that collectively achieve the neutralization of xenobiotics. Two major detoxification systems have evolved. One is the non-catalytic multidrug resistance system (MDR) that utilises P-glycoproteins, large integral proteins belonging to the superfamily of "traffic ATPases", to transport harmful compounds to the extracellular space, at the expense of ATP hydrolysis (Endicott & Ling, 1989). The other detoxification system requires the metabolic activities of several enzyme systems that collectively convert lipophilic harmful compounds into hydrophilic, excretable compounds. The latter system is by far the most complex and is the one studied in this investigation. Drug metabolism is normally divided into two phases, **phase I** (or functionalisation reactions) and **phase II** (or conjugative reactions). Generally speaking, phase I reactions are considered to be the preparation of the drug which is then acted on by phase II enzymes. For example a phase I reaction might produce or uncover chemically reactive groups, such as -OH, $-NH_2$, -SH, -COOH. etc. (Guengerich, 1990).

The chemical reactions associated with phase I and phase II metabolism are shown in Table 1.1, and a simplified overview of xenobiotic metabolism is given in Figure 1.1.

Table 1.1 Reactions classed as Phase I or Phase II metabolism

Phase I	Phase II
Oxidation	Glucuronidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacetylation	Amino acid conjugation
Isomerisation	Glutathione conjugation
	Fatty acid conjugation
	Condensation

Reproduced from Gibson & Skett (1994).

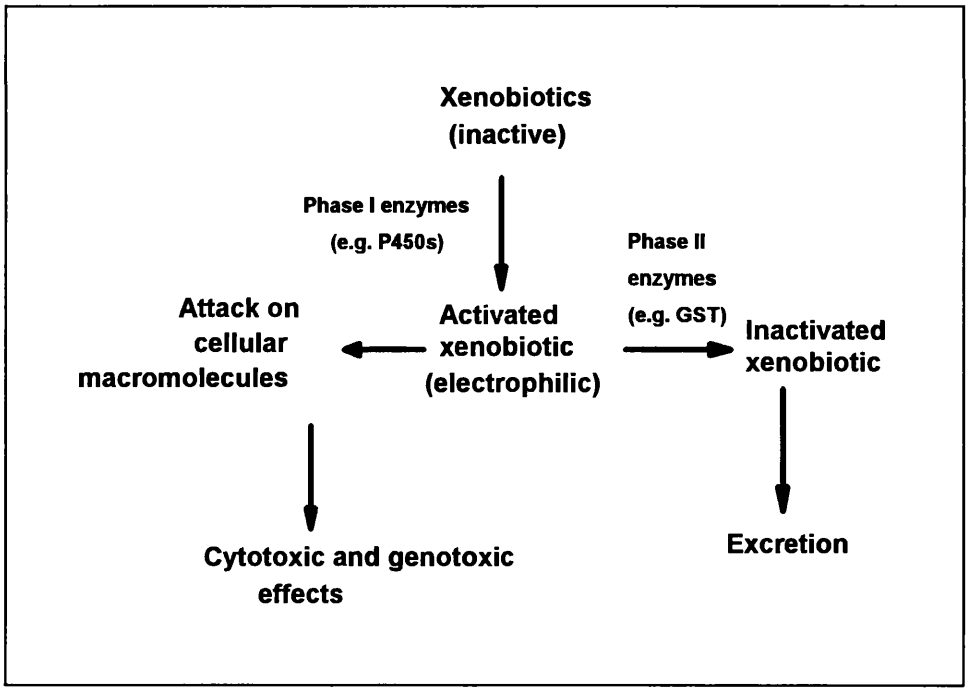


Fig. 1.1 Simplified overview of xenobiotic metabolism

1.2 Enzymology of Phase I and Phase II metabolism

In quantitative terms, the liver is the main organ responsible for both phase I and phase II metabolism, although drug metabolising enzymes have also been found in lung, kidney, gut, skin and other tissues (Connelly & Bridges, 1980; Kappus, 1986). Studies on isolated sub-cellular hepatocyte compartments and isolated enzymes have shown that the endoplasmic reticulum and the cytosol are the two most important sub-cellular organelles in drug metabolism. Phase I enzymes are almost exclusively localised in the endoplasmic reticulum (ER), whereas phase II enzymes are localised in the cytosol (Jakoby, 1980). However, exceptions to this general statement exist in the form of cytosolic epoxide hydrolase (phase I enzyme; Schladt et al., 1988) and of microsomal glutathione transferase (phase II enzyme; Morgenstern et al., 1985). Moreover, UDP-glucuronosyl transferases (phase II enzymes) are localized in the ER (Tephly & Burchell, 1990). Most of the phase I reactions are catalysed by the cytochrome P450-dependent mixed-function oxidase system.

1.2.1 Cytochrome P450-dependent mixed-function oxidase system

Cytochromes P450 (P450s) are the terminal oxidases of an electron transfer system known as the P450-dependent mixed function oxidase system (Lu & Coon, 1968; Fig. 1.2). In addition to P450s, this system is comprised of NADPH dependent cytochrome P450 reductase (P450 reductase), NADH dependent cytochrome b_5 reductase (b_5 reductase), and cytochrome b_5. In this system electrons are donated from NAD(P)H to a flavoprotein reductase (P450 reductase and/or b_5 reductase) (Gunsalus & Sligar, 1977). Reducing equivalents are then passed to clusters of cytochromes P450 either directly or via Cytochrome b_5 (Fig. 1.3 and 1.4). The general reaction catalysed by this system

involves the insertion of a single oxygen atom into the substrate and it has the following stoichiometry (Guengerich, 1990; R is the substrate):

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

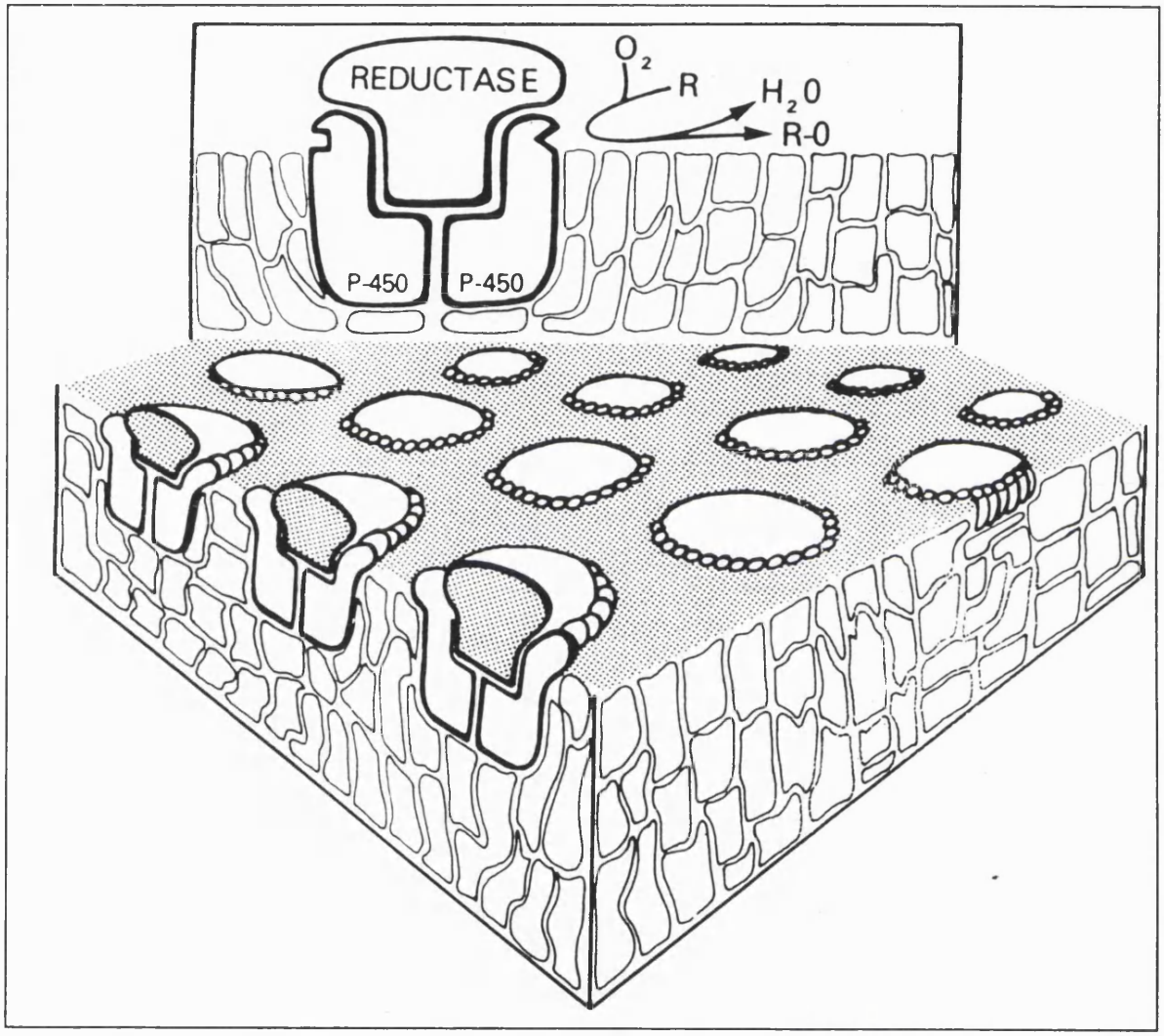


Fig. 1.2 Three-dimensional representation of the mixed function oxidase system embedded in the endoplasmic reticulum. "R" is the substrate and "reductase" is P450 reductase. Reproduced from Nebert & Gonzalez (1987).

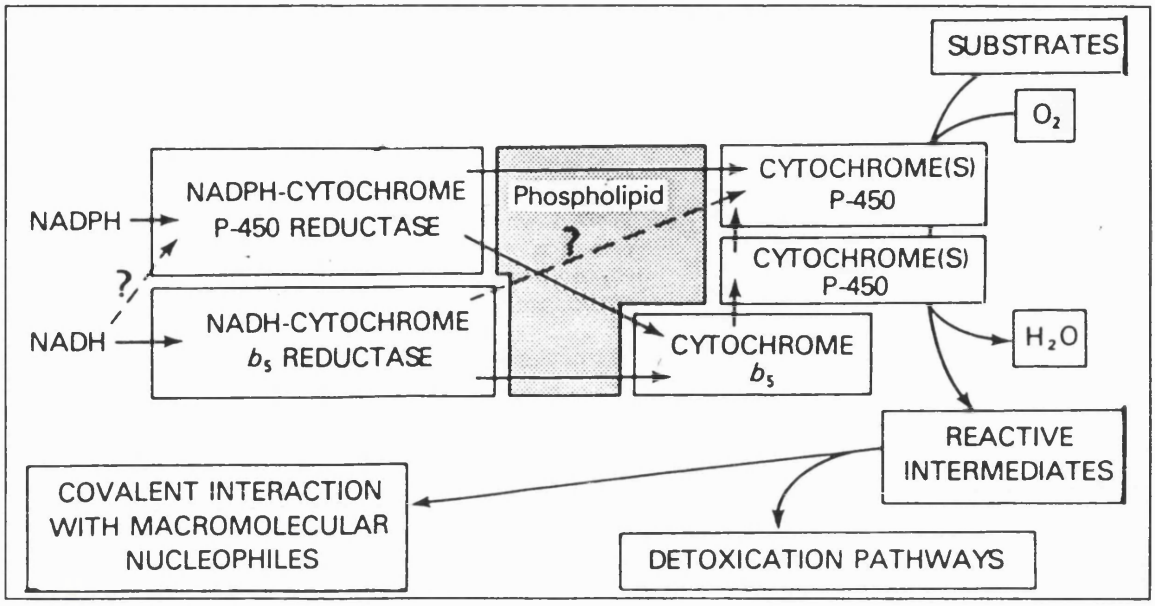


Fig. 1.3 Schematic representation of the electron transport pathway in the mixed function oxidase system. Reproduced from Gonzalez et al. (1986)

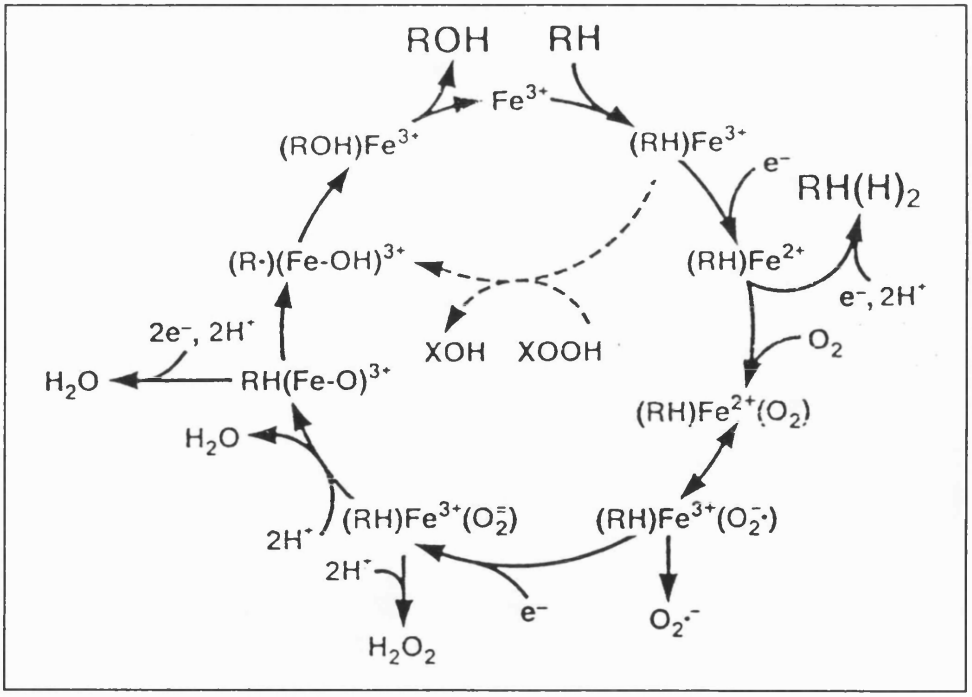


Fig. 1.4 Scheme for mechanism of action of cytochrome P450. "RH" denotes the substrate and "ROH" the oxygenated product. Reproduced from Porter & Coon (1991).

As well as hydroxylation reactions, both aliphatic and aromatic, the mixed-function oxidase system also catalyses epoxidation, N-dealkylation, O-dealkylation, S-dealkylation, oxidative deamination, N-oxidation, S-oxidation, phosphothionate oxidation and dehalogenation (Fig. 1.5).

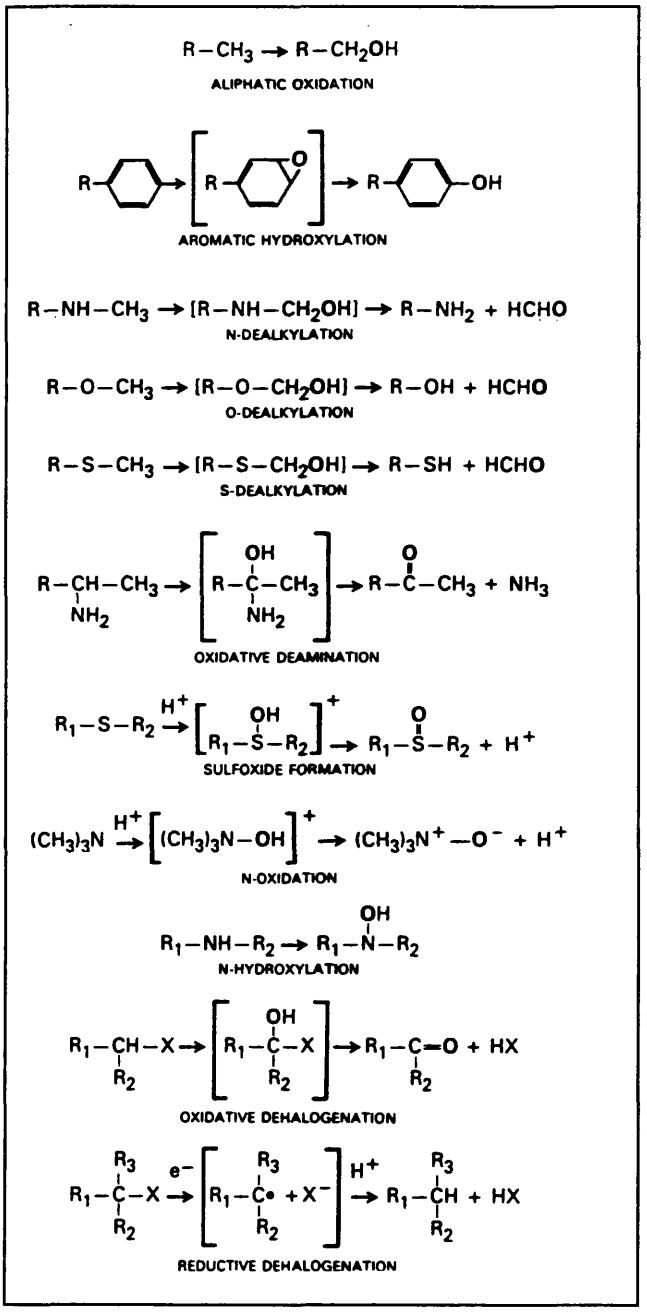


Fig 1.5 Examples of the chemical reactions catalysed by the mixed function oxidase system. Reproduced from Nebert & Gonzalez (1987).

1.2.1.1. Components of the cytochrome P450-dependent mixed-function oxidase system.

I) Cytochromes P450: multiplicity and nomenclature

Cytochromes P450 are classified as haemoproteins, with iron protoporphyrin IX as the prosthetic group (Fig. 1.6). They belong to a very large family of closely related isoenzymes that have molecular weights of approximately 45,000 - 55,000. The haem is non-covalently bound to the apoprotein and the name Cytochrome P450 derives from the fact that the cytochrome (or pigment) shows a Soret peak at 450 nm, when reduced and complexed to carbon monoxide (Omura & Sato, 1964; Omura & Sato, 1964b). The haemoprotein serves as both the oxygen- and substrate binding locus for the mixed function oxidase reaction.

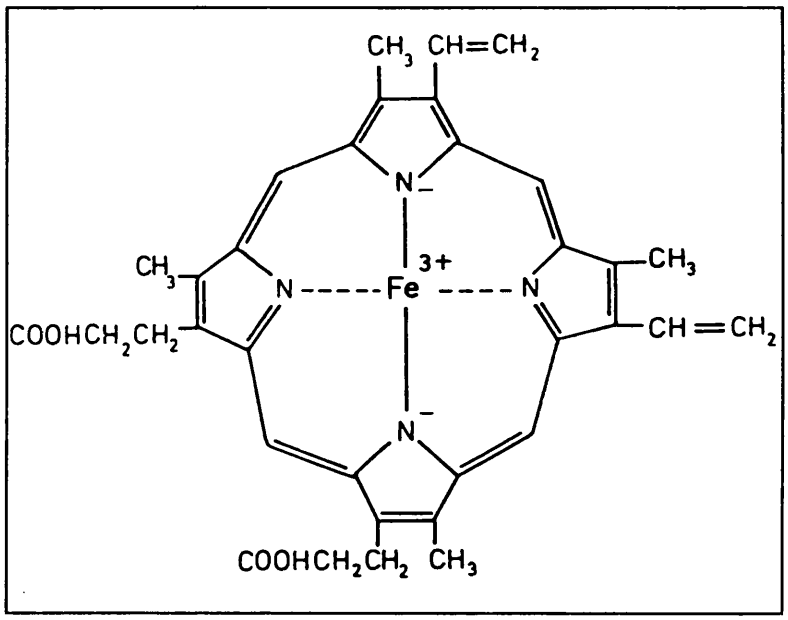


Fig. 1.6 Structure of ferric Protoporphyrin IX

After several laboratories had managed to successfully purify cytochromes P450 to homogeneity, it became evident that different forms of cytochromes P450 exist, not only across species but also within an organism. The large number of cytochromes P450 characterised and the large number of laboratories involved have contributed in the past to the confusing nature of cytochromes P450 nomenclature: often the same cytochrome P450 was designated with different names. However, with the advent of molecular biology, an explosion in the number of available cDNAs has lead to a rapid accumulation of amino acid sequences. Cytochromes P450 are classified into gene families and subfamilies (Nebert et al., 1987). In this system two cytochromes P450 belong to different families if their primary amino acid sequence shares an identity of $\leq$ 36 %. Within a gene family, cytochromes P450 can be further divided into sub-families, where a protein of one subfamily shares 40 to 65 % sequence identity with a protein of another subfamily (i.e. two cytochromes P450 belong to the same gene subfamily if $\geq$ 65 % of their amino acids are identical). The use of the italicized root symbol "*CYP*" ("*Cyp*" for the mouse) to denote cytochrome P450 genes is recommended in the latest update to the nomenclature system (Nelson et al., 1993). The non-italicized root symbol instead is used to indicate cytochromes P450 proteins, mRNAs and cDNAs. An Arabic number is used to designate a cytochrome P450 gene family, a letter to indicate the subfamily, and an Arabic number is used to represent the individual gene. With mouse genes or cDNAs, the final number is generally preceded by a hyphen. For example, "*CYP1A1*" ("*Cyp1a-1*" for the mouse gene) indicates the cytochrome P450 gene 1, belonging to the subfamily A of the family 1. If no second subfamily or second gene exists, the subfamily and gene number can be omitted, e.g. *CYP5* or *CYP19.*

The very large number of cytochromes P450 [more than 200 different cytochromes P450 have been cloned and/or isolated (Nelson et al., 1993)] and the equally large number of chemical reactions they catalyse, makes this enzyme system the most versatile biological catalyst known. Cytochromes P450 are involved in the oxidative metabolism

of steroids (cytochromes P450 involved in steroidogenesis are localised in the mitochondria), fatty acids, leukotrienes, prostaglandins, pheromones, plant metabolites, and many drugs, environmental pollutants and chemical carcinogens [reviewed in Nebert & Gonzalez (1987); Gonzalez (1990); Porter & Coon (1991)].

Studies using reconstituted systems of purified cytochromes P450 established that different forms of P450 can exhibit either highly specific or less specific overlapping substrate specificities (Guengerich, 1987). For example, a single form of P450 can have a low K_m for one substrate and a high K_m for another substrate. Moreover, cyclic compounds, such as testosterone, can be specifically hydroxylated at different ring positions by different P450 isoenzymes.

I.I) Cytochromes P450: overview of the gene superfamily and of the regulation of the expression of its genes

At the end of December 1992 (Nelson et al., 1993), the cytochrome P450 gene superfamily comprised 36 families, of which 12 are mammalian. These twelve families comprise twenty-two mammalian subfamilies, of which seventeen and fifteen have been mapped to the human and murine genome, respectively. Cytochromes P450 that belong to families 1 to 4 are involved in drug metabolism. Those belonging to the remaining families are primarily involved in steroidogenesis.

Although catalytic activity of the mixed function oxidase system requires the presence of the flavoprotein and lipids, it is the haemoprotein cytochrome P450 that confers substrate specificity to the system. Genetic differences in activity levels have been shown to be solely associated with the cytochrome P450 protein (Haugen et al., 1976). Many factors (these include age, sex, dietary components, health status, ontogenic development, hormones, and exposure to xenobiotics) influence the expression pattern of individual cytochromes P450 (Gonzalez, 1989). Variations in the pattern of expression of cytochromes P450 explain, at least in part, the wide qualitative and quantitative

differences observed among species and individuals with respect to their drug metabolising capacity. In humans, for example, the debrisoquine hydroxylation polymorphism has been well defined and linked to *CYP2D6* polymorphism (reviewed in Cholerton et al., 1992). The number of people that lack the ability to metabolise debrisoquine (poor metabolizers) ranges from 5 % to 10 % in Caucasians (Fig. 1.7). In Oriental subjects, however, individuals that cannot metabolise debrisoquine have yet to be identified (Eichelbaum & Gross, 1990).

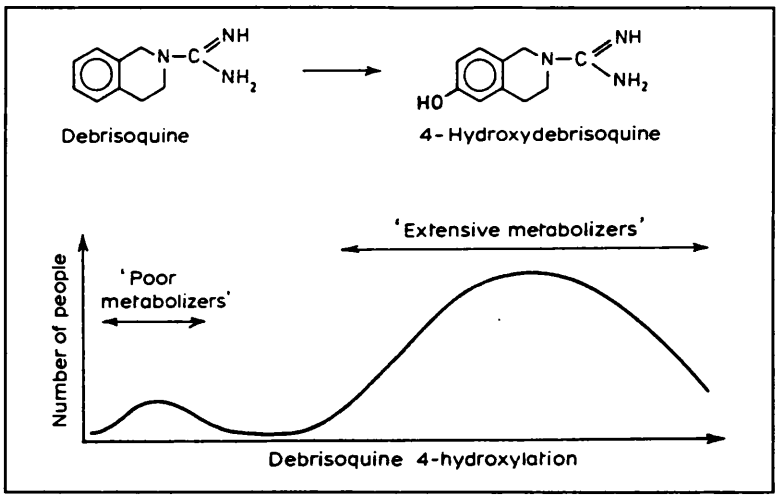


Fig. 1.7 The distribution of the rate of metabolism of debrisoquine in the Caucasian population. Reproduced from Gibson & Skett (1994).

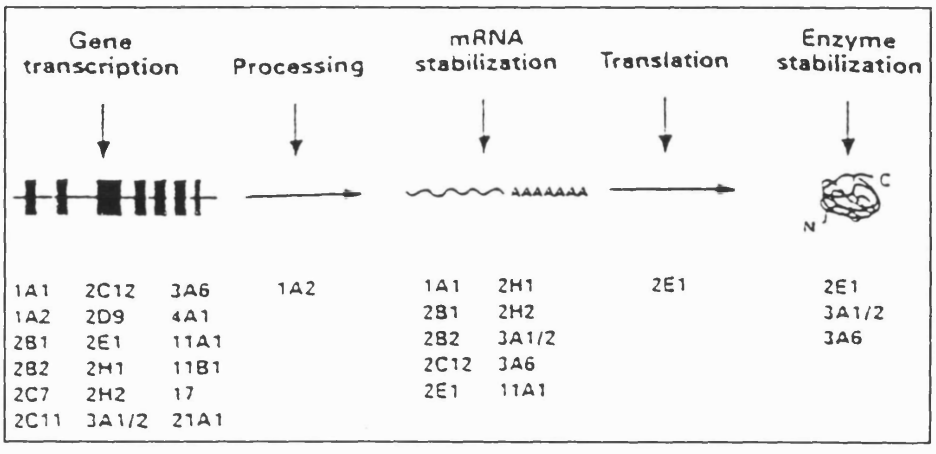


Fig. 1.8 Multiplicity in the regulation of cytochrome P450 expression. Reproduced from Porter & Coon (1991)

Considering the large number of cytochrome P450 genes it is not surprising that a considerable diversity exists in the mechanisms that regulate the expression of these genes [Fig. 1.8; reviewed in Nebert & Gonzalez (1987); Gonzalez (1989); Gonzalez et al. (1993)]. Transcriptional regulation is by far the most common. However, in some cases, mRNA stabilisation (Dalet et al. 1988), RNA processing (Kawajiri et al., 1986), regulation of translation (Johansson et al., 1988), and protein stabilisation (Watkins et al., 1986) also play a role. Sometimes the same gene is regulated by more than one mechanism (e.g. *CYP2E1;* Johansson et al., 1988).

The expression of some cytochrome P450 genes is either switched-on or augmented by a variety of drugs and other xenobiotic chemicals. This phenomenon may have evolved as an adaptive response to chemical stress and is generally referred to as "induction" (reviewed in Okey, 1990). Because of the broad substrate specificity of cytochrome P450 enzymes, induction has profound implications in pharmacology, toxicology and carcinogenesis (Okey et al., 1986). Many xenobiotic chemicals, in fact, stimulate not only their own metabolism, but also the metabolism and clearance of

unrelated compounds. It has been repeatedly demonstrated that humans treated with barbiturates show significant increases in the clearance rates for several other drugs, including antipyrine, phenytoin, oral contraceptives and warfarin (Park & Breckenridge, 1981). Moreover, it is well-recognised that certain cytochromes P450 can convert relatively unreactive parent compounds into chemically-reactive intermediates that are either toxic or carcinogenic (Conney, 1982).

Five prototype inducers have been identified: 3-methylcholanthrene (3-MC), phenobarbital (PB), dexamethasone (DEX), ethanol, and chlofibrate (CHLO).

3-methylcholanthrene and a number of other compounds that are generally defined as polycyclic aromatic hydrocarbons (PAH; the structures of some these compounds are shown in Fig 1.10), including the carcinogens benzo[a]pyrene and benz[a]anthracene, are particularly good inducers of members of the **CYP1** family (Conney, 1967). The most potent inducers in this category are highly toxic halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (Poland & Knutson, 1982). The CYP1A subfamily consists of two members, CYP1A1 and CYP1A2, in most species examined to date. CYP1A1 is particularly active against polycyclic aromatic hydrocarbons, such as benzo[a]pyrene (Guengerich et al., 1982; Ryan et al., 1982). CYP1A2 instead has a high catalytic activity towards arylamines (Johnson et al., 1980). Both CYP1A1 and CYP1A2 have overlapping substrate specificities. Because *CYP1A2* is not expressed in cell culture, the molecular mechanism(s) involved in the regulation of the expression of this gene is not well understood. *CYP1A2* is constitutively expressed in liver but its transcription rate is augmented a few fold by inducer administration (Gonzalez et al., 1984). In contrast, *CYP1A1* expression is detected both in animals (Negishi & Nebert, 1981) and cultured cells (Israel & Whitlock, 1984) only after treatment with inducers. The molecular mechanism involved in the induction of *CYP1A1* gene expression is fairly well understood. The inducer binds to the ligand-binding domain of the *Ah receptor*

(Aromatic hydrocarbon receptor; reviewed in Swanson & Bradfield, 1993). Despite early predictions that the *Ah* receptor might belong to the steroid receptor *erb*-like superfamily (Denison et al., 1986), recent cloning studies (Burbach et al., 1992; Ena et al., 1992) have illustrated that it is unique among known receptors. The *Ah* receptor, in the absence of the inducer, is located in the cytoplasm in its inactive form, complexed to heat shock protein 90 (hsp90; Pongratz et al., 1992). Upon binding of the inducer to the receptor, the hsp90 protein is displaced, and the Ah receptor then interacts with a protein known as the *Ah receptor nuclear translocator* (Arnt; Hoffman et al., 1991). This complex is now capable of translocating to the nucleus and binding to specific *cis*-acting regulatory elements located upstream of *CYP1* genes (Reyes et al., 1992; Fig. 1.9). These elements have been variously referred to as Xenobiotic Regulatory Element (XRE), Drug Regulatory Element (DRE) and Ah regulatory element (AhRE). As a consequence of Ah/Arnt XRE interaction, strong transcription of *CYP1A1* is initiated (Nebert & Jones, 1989; Hoffman et al., 1991). This interaction is also thought to mediate inducer-dependent increases in *CYP1A2* transcription (Pasco et al., 1988; Okey, 1990). Other factors involved in *CYP1A1* regulation have been suggested based on reporter gene transfection assays and analysis of variant hepatoma cell lines. A basal transcription element (BTE) has been found in the 5'-flanking sequence of the *CYP1A1* gene (Fujii-Kuriyama et al., 1992). This regulatory element appears to be involved in the constitutive expression of a variety of cytochrome P450 genes, including *CYP2B2* (Jaiswal et al., 1987) and *CYP2E1* (Ueno & Gonzalez, 1990). A protein that is expressed in a variant mouse hepatoma cell line has been identified and shown to inhibit transcription by binding to the AhRE (Watson et al., 1992).

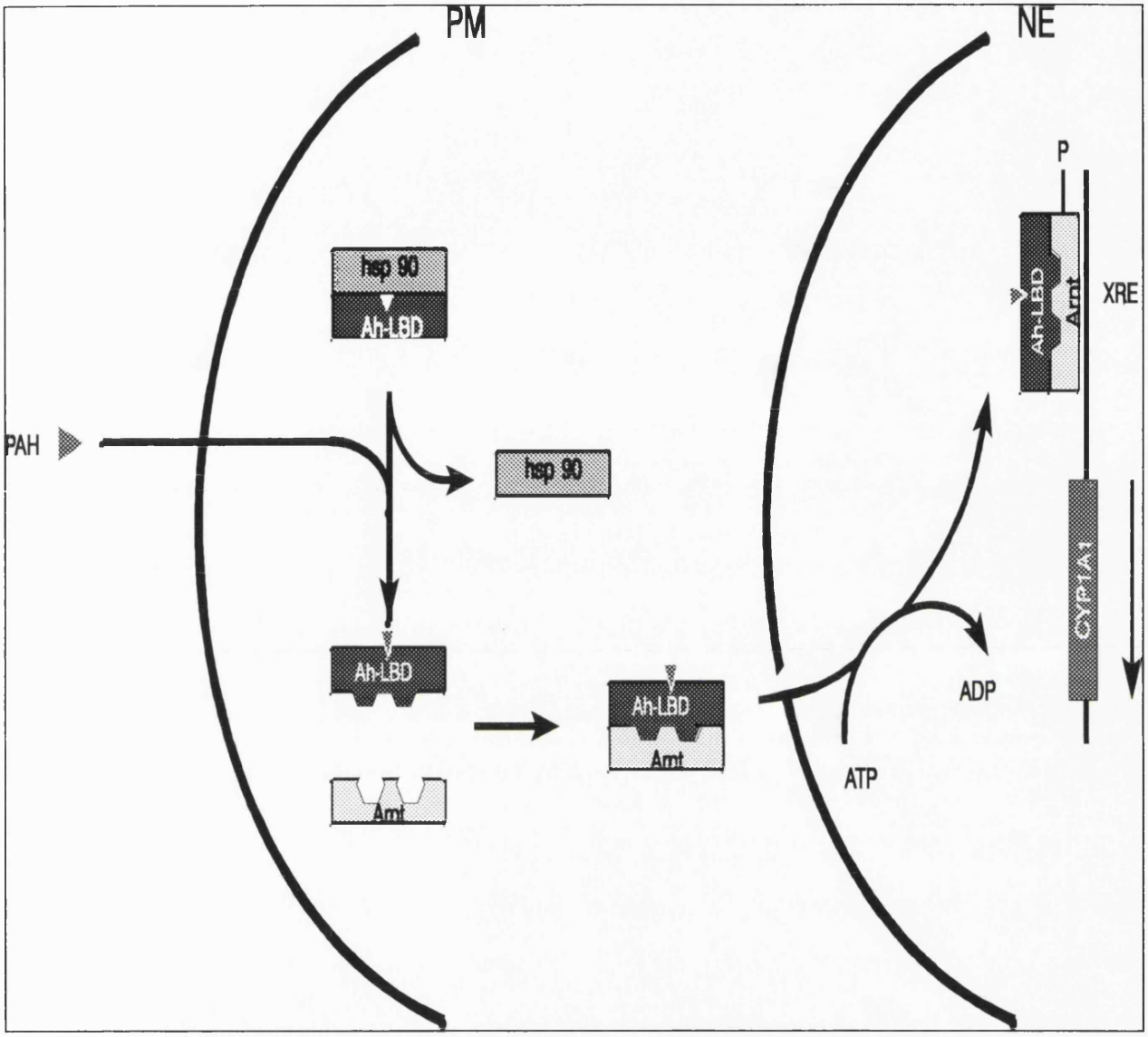


Fig. 1.9 Scheme depicting current knowledge of the regulation of the *CYP1A1* gene. The ligand is shown as a triangle. PM: plasma membrane; NE: nuclear envelope; PAH: polyaromatic hydrocarbons; Ah-LBD: Aromatic hydrocarbons-ligand-binding-domain; Arnt: Ah receptor nuclear translocator; XRE: xenobiotic response element. Reproduced from Gonzalez (1993).

Phenobarbital (PB, Fig. 1.10), a barbiturate once used in the treatment of epilepsy, and a large number of structurally unrelated compounds termed "PB-like" inducers (the chemical structures of some of these compounds are shown in Fig. 1.10) can elevate the expression of individual members of the **CYP2A**, **2B**, **2C**, and **3A** subfamilies, both in laboratory animals (Waxman et al., 1985), and in cultured rat and human hepatocytes (Akrawi et al., 1993; Morel et al., 1990). Moreover, many other enzymes which contribute to foreign compound metabolism, including P450 reductase (Shephard et al., 1983), epoxide hydrolase (Thomas et al., 1981; Guengerich, 1982b), aldehyde dehydrogenase (Dunn et al., 1989), UDP-glucuronosyl transferase (Henry & Gasciewicz, 1987) and several glutathione-S-transferases (Pickett et al., 1984) are also inducible by PB. The effects of PB also include proliferation of smooth endoplasmic reticulum (Remmer & Merker, 1963), stimulation of liver weight gain (Conney, 1967), liver tumour promotion (Schulte-Hermann, 1974), and a general stabilisation of liver microsomal protein (Omura, 1979). Phenobarbital induction of drug-metabolising enzymes is reviewed in Pickett & Lu (1989); Okey (1990); and Waxman & Azaroff (1992). To date, the molecular mechanisms involved in PB induction remain unknown.

In rat, two cytochromes P450, known as CYP2B1 and CYP2B2, have been well characterized (Guengerich et al., 1982; Ryan et al., 1982). In untreated rat liver CYP2B2 is present at a low but measurable amount, whereas CYP2B1 protein expression is at least 5-10-fold lower and often undetectable. However, the expression of both these cytochrome P450 proteins is elevated dramatically above basal levels (typically, 50-100-fold for CYP2B1 and 20-fold for CYP2B2) upon exposure of rats to phenobarbital (Thomas et al., 1981; Phillips et al., 1983; Christou et al., 1987). In contrast, members of the CYP2A and 2C subfamilies are only modestly induced by phenobarbital (Leighton & Kemper, 1984; Ryan & Levin, 1990). Much effort has therefore been devoted to understanding the mechanisms involved in the induction by phenobarbital of CYP2B1 and CYP2B2. These two cytochromes P450 show similar broad, overlapping specificity

towards a large number of lipophilic drugs and steroidal substrates, including benzphetamine (Guengerich et al. 1982; Waxman & Walsh, 1982) and testosterone (Waxman et al., 1983). CYP2B1 usually exhibits higher activity than CYP2B2 towards certain substrates (Ryan et al., 1982). These two proteins exhibit 97 % cDNA-deduced amino acid similarity (14 substitutions in 491 amino acids, Suwa et al., 1985) and have distinct chromatographic and electrophoretic properties. The genes that code for these two enzymes, *CYP2B1* and *CYP2B2*, are closely linked on rat chromosome 1 (Rampersaud & Walz, 1983), contain nine exons, and only forty nucleotide differences exist between the two mRNAs (Fujii-Kuriyama et al., 1982). The induction by phenobarbital of the CYP2B1 and CYP2B2 proteins is tissue-specific (Christou et al., 1987; Traber et al., 1990). Maximal expression and induction of these two proteins occur in the liver. In the small intestine, CYP2B1, but not CYP2B2 is induced by PB. Whereas in lung and testes CYP2B1 appears to be constitutively expressed but is not PB-inducible. Developmental factors also play a role in the regulation of CYP2B1 and CYP2B2 expression. Both mRNAs are expressed at very high constitutive levels in foetal liver, where they can be transplacentally induced as early as day 15 of gestation (Giachelli & Omiecinski, 1986; Omiecinski et al., 1990). Moreover, the responsiveness to PB of these two cytochromes P450 in adult rats is greater in males than it is in females. The major regulating factor appearing to be the suppressive influence of serotonin and its continuous pattern of expression in female rats, as shown by studies performed *in vivo* (Yamazoe et al., 1987) and by experiments carried out on cultured cells (Schuetz et al., 1990; Waxman et al., 1990). Within a single organ, distinct regions or cell types may express *CYP2B* genes at different levels. In liver, for example, *in situ* hybridization studies have revealed that phenobarbital-inducible CYP2B1 and CYP2B2 mRNAs are only expressed in parenchymal cells that are located in the centri-lobular and mid-zonal regions of the hepatic lobule (zonation of the liver is reviewed in Gebhardt, 1992). In contrast, CYP2B1/2 mRNAs are undetectable in parenchymal cells

surrounding the periportal tract (Traber et al., 1989; Chianale et al., 1986). These results have also been recently confirmed at the protein level by immunohistochemistry (Buhler et al., 1992). Indeed, this characteristic expression pattern of *CYP2B* genes in the liver lobule has been suggested to be due to a regiospecific suppression by growth hormone in the periportal region (Oinonen et al., 1994).

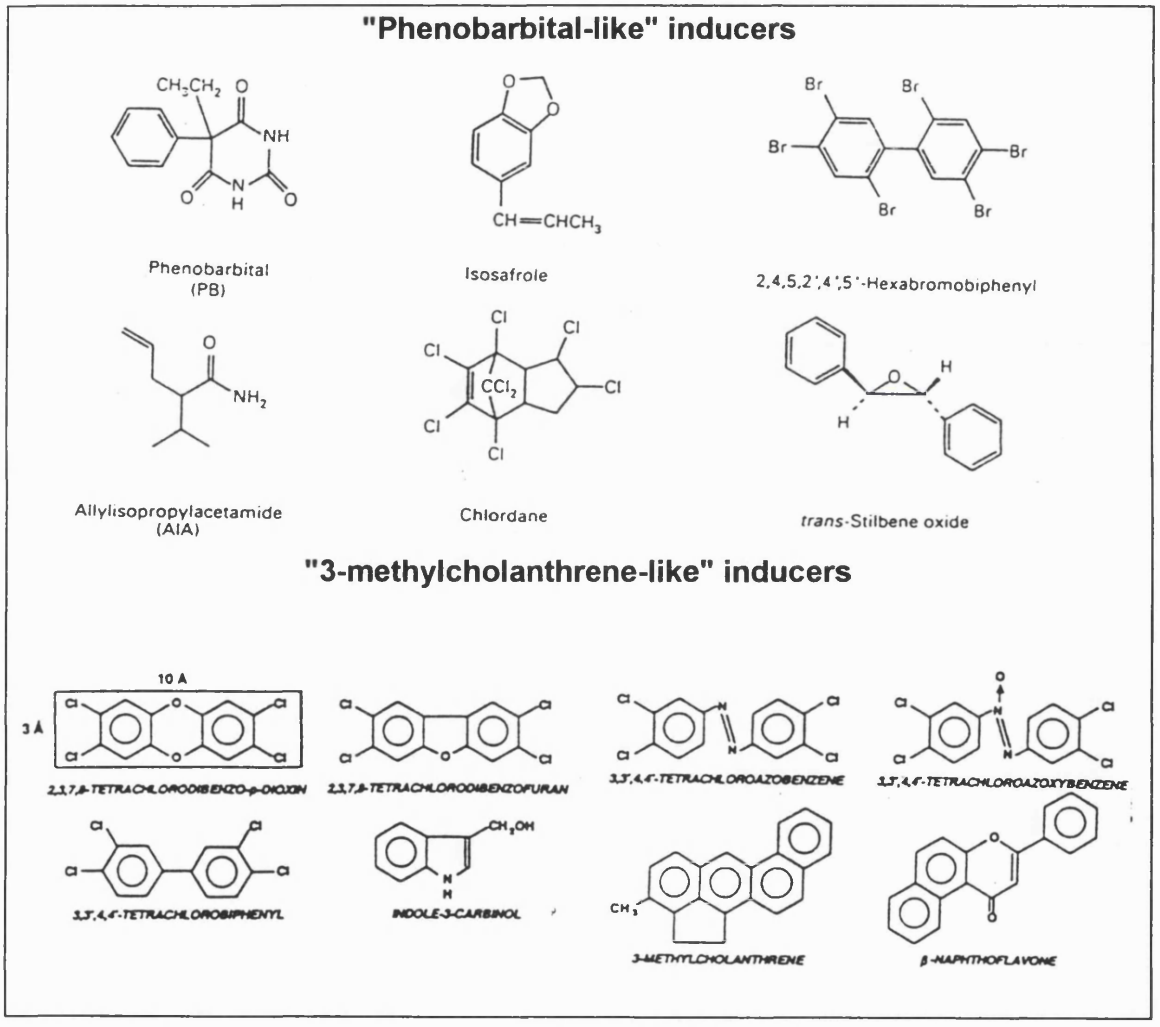


Fig 1.10 Chemical structures of some inducers of cytochrome P450.

The induction by phenobarbital of CYP2B1 and 2B2 was shown to be the direct consequence of an increase in the steady-state levels of their mRNAs (Phillips et al., 1981). Run-on transcription analyses further demonstrated that the increase in steady-state mRNA levels was due to increased transcription of the corresponding *CYP2B* genes. Transcription initiation and not transcription elongation was shown to be the most likely regulated step during PB induction (Pike et al., 1985). Other regulatory mechanisms such as gene amplification and gene rearrangement have been ruled out. The transcription initiation site of both *CYP2B* genes is 33 bp. upstream from the translation initiation site. A possible TATA sequence was found 27 bases upstream of base +1 (Fujii-Kuriyama et al., 1982; Suwa et al, 1985) and both genes are characterised by an alternating purine/pyrimidine sequence located at -255. This sequence differs in length between *CYP2B1* and *CYP2B2* - $(CA)_5$ for *CYP2B1* and $(CA)_{19}$ for *CYP2B2* - and it has been proposed to form a Z-helical structure that may be of regulatory significance (Suwa et al., 1985). Gel retardation and DNase I footprinting experiments have recently identified two DNA sequences located between -183 to -199 and -31 to -72 of the *CYP2B2* gene promoter that bind rat liver nuclear proteins that are enriched or activated *in vivo* by phenobarbital (Shephard et al., 1994). Putative Pit-1 regulatory elements that may have a role in the regulation by growth hormone of this gene have also been identified in the 5'-flanking region and a glucocorticoid response element (GRE) located between -1357 and -1343 has been shown to confer dexamethasone inducibility to heterologous genes (Jaiswal et al., 1990).

Promoter mapping experiments designed to identify possible PB-responsive elements have been notoriously difficult to perform due to the fact that *CYP2B* gene expression, and its induction by phenobarbital, is characteristically lost in cultured hepatocytes and almost absent in cell lines (Sirica & Pitot, 1980). However, some progress has been made in birds and bacteria. *Trans*-activation experiments using plasmid constructs containing the CAT gene fused to upstream sequences of the *CYP2H1* gene

have demonstrated the existence of a *cis*-acting element that is responsive to phenobarbital in chicken embryo primary hepatocyte cultures. A 4.8 kb fragment, located 1.1 kb upstream of the transcription start site, was shown to undergo a fourteen-fold induction in the presence of PB (Hahn et al., 1991). The precise sequence responsible for this effect, however, has not been defined, nor has a transcription factor or a receptor been identified.

In bacteria, a region located upstream of the *CYP102* gene was found to confer transcriptional activation in the presence of phenobarbital (Shaw & Fulco, 1992). This activation of transcription appears to be due to the release of a repressor protein, named Bm3R1, that has been shown to bind to a palindromic sequence located immediately upstream of the *CYP102* gene (Wen et al., 1989). It is still unclear whether phenobarbital acts directly, as depicted in Fig. 1.11, to displace the repressor from the operator. It should be interesting to determine whether P450 genes in higher eukaryotes are also activated via a phenobarbital-mediated displacement of a repressor protein. Interestingly, a putative negative regulatory element has been reported to be located between -800 and -20 kb of the rat *CYP2B2* gene. Moreover, it was reported that at least 20 kb of this gene 5'-flanking region were necessary to elicit an induction response in transgenic mice (Ramsden et al., 1993). This observation could be however a result of positional effects exerted by sequences flanking the transgene at the site of integration. A shorter (1.4 kb) fragment of the 5'-flanking region of the *CYP2B2* gene has been reported to be sufficient for a phenobarbital-dependent three-fold increase in reporter gene activity in rat hepatoma FGC4 cells (Shaw et al., 1993).

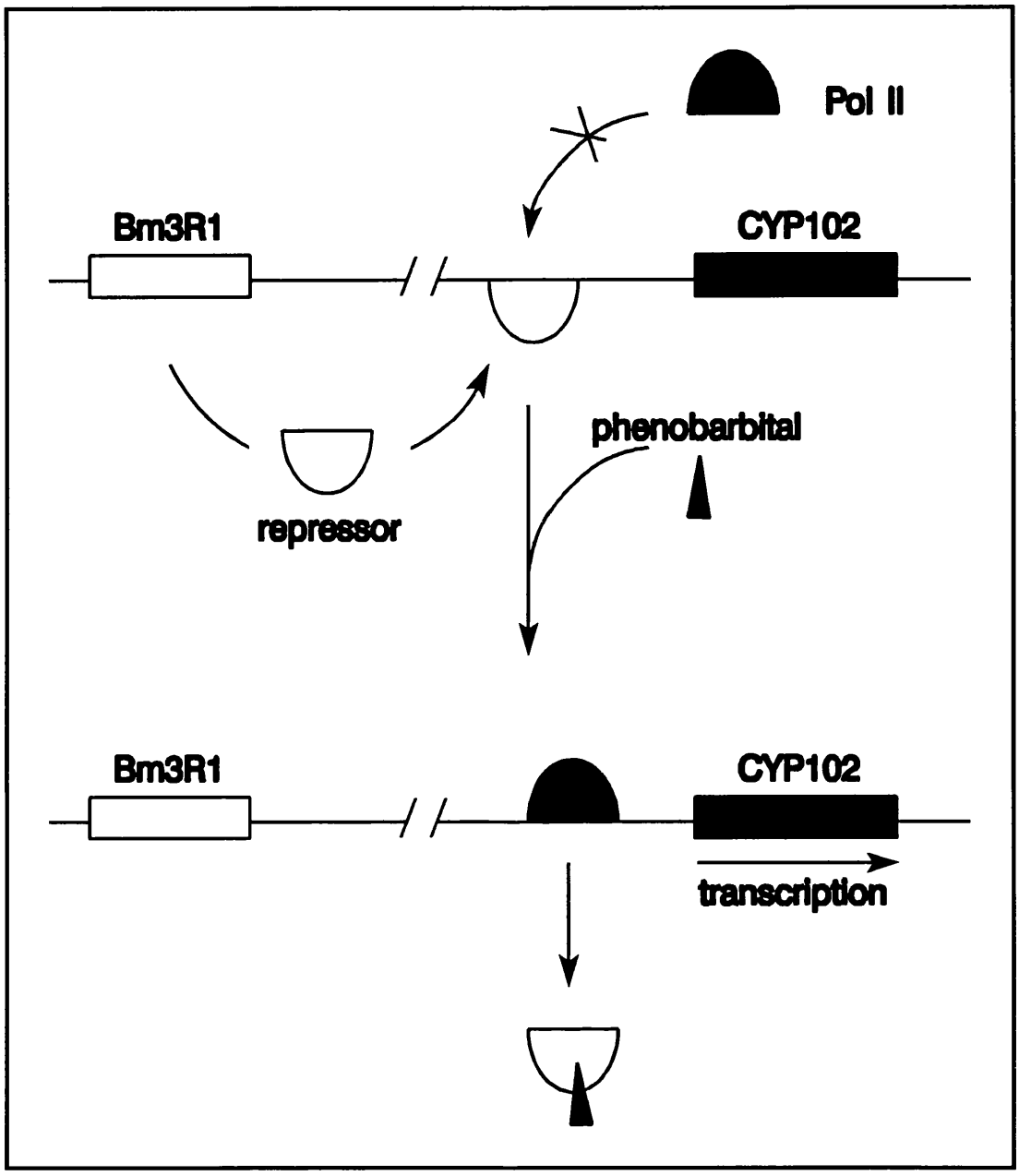


Fig. 1.11 Regulation of *CYP102* by phenobarbital. Reproduced from Gonzalez (1993)

Although phenobarbital is the prototype inducer, many other "phenobarbital-like" compounds have been identified. These include isosafrole, *trans*-stilbene oxide, allylisopropylacetamide, chlordane and other organochlorine pesticides, various non-planar halogenated biphenyls and carcinogens such as acetylaminofluorene (Thomas et al., 1981; Dannan et al., 1983; the chemical structures of some of these compounds are shown in Fig. 1.10). The major mystery in the phenobarbital induction system is how the cell recognises these inducers and how the information is conveyed to the transcriptional machinery. No quantitative structure activity relationships could be established among PB-like inducers. All these compounds appear to lack any common structural feature, other than their general lipophilicity. Nonetheless, several experimental observations have led to the hypothesis that an intracellular phenobarbital receptor exists: a) all PB-like inducers are lipophilic and hence are intrinsically able to enter cells; b) all inducers stimulate expression of essentially the same subset of P450 genes; c) the phenobarbital response shows a saturable dose response curve and is tissue-specific; d) the characteristic low potency of PB-like inducers predicts that if a receptor does exist it should have a relatively low affinity for the ligand and could not be detected by conventional receptor assay procedures. Alternative regulatory mechanisms have also been suggested. These include the possibility that PB and related compounds may stimulate *CYP2B* expression by causing general changes in cellular physiology, or direct interaction of the inducer with P450 enzymes. The latter hypothesis predicts that the inducer interacts with one or more constitutively expressed P450 enzymes. It is possible that the inducer may interact with CYP2B2 itself, since it is expressed at low, but detectable levels in uninduced rat liver (Christou et al., 1987). Interaction of the substrate with the enzyme would lead to inhibition of the metabolic activity of this P450 towards an endogenous substrate that, if unmodified and/or accumulates to sufficiently large amounts, has the ability to stimulate transcription of *CYP2B* genes. A steroid could be a likely candidate for such an endogenous substrate. Indeed, recent evidence could be

interpreted to support this hypothesis (Shaw et al., 1993). A compound known as RU486, a strong glucocorticoid/progesterone antagonist, was shown to inhibit the transcriptional activation, in FGC4 cells, of a *CYP2C6*-promoter/luciferase construct. These findings however do not exclude the possibility that RU486 may prevent induction by inhibiting the interaction of PB with a specific receptor, in a *competitive* or *non-competitive* fashion. Indeed if, as in the case of the Ah receptor, a nuclear translocation step is required in PB induction, it could be predicted that RU486 may prevent nuclear translocation of the receptor, perhaps by interfering with an Arnt-like molecule.

It is interesting to observe that phenobarbital has been shown to stimulate accumulation of *CYP3A* mRNAs both *in vivo* and *in vitro* (Marie & Cresteil, 1989; Sidhu et al., 1993). The expression of these genes is strongly induced by glucocorticoids such as dexamethasone or pregnenolone 16α carbonitrile.

Only a single ***CYP2E*** gene has been identified in man (Umeno et al., 1988) and rat (Umeno et al., 1988b). In contrast, two genes have been identified in rabbit, both of which are expressed (Khani et al., 1988). The prototype inducer of ***CYP2E*** genes is **ethanol**. In addition, several other volatile (and some non volatile) compounds, including acetone, isoniazid, isopropanol, trichloroethylene, pyrazole, methyl pyrazole induce CYP2E1 (Okey, 1990). Interestingly, CYP2E1 is also induced by fasting and diabetes, possibly via increased levels of ketone bodies (Bellward et al., 1988). In contrast to most other members of the CYP2 family, CYP2E1 is not influenced by PB, even though ethanol has been shown to induce PB-inducible enzymes both *in vivo* and *in vitro* (Johansson et al., 1988; Sinclair et al., 1991). Substrates for the enzyme include ethanol itself, aniline, ketones (including acetone), and nitrosamines. In particular it has been shown to increase the toxicity of the procarcinogen *N*-nitrosodimethylamine (Koop et al., 1982; Casazza et al., 1984; Yang et al, 1990).

Multiple mechanisms appear to be involved in CYP2E1 regulation (Fig. 1.8). Induction of these genes appears to occur via an increase in protein and mRNA

stabilisation, rather than via increased transcription (Gonzalez et al., 1988). The constitutive expression of the *CYP2E1* gene, at least *in vitro*, was found to be controlled by a "liver-enriched" transcription factor, HNF-1α (Hepatic Nuclear Factor-1α) (Ueno & Gonzalez, 1990; liver-specific regulation is reviewed in De Simone & Cortese, 1991; Sladek & Darnell, 1992). This observation is worth mentioning since, like *CYP2B* genes, *CYP2E1* is extinguished in primary cultures of rat hepatocytes, together with the *HNF-1 α* gene (Gonzalez et al., 1993). The latter gene is under the regulation of another liver-enriched transcription factor, HNF-4, suggesting a transcriptional hierarchy in *CYP2E1* expression and possibly in other P450 genes (Kuo et al., 1992).

Cytochromes P450 belonging to the *3A* subfamily were first identified by virtue of the fact that they are induced by the prototypic compound **pregnenolone-16α-carbonitrile** (PCN). The first enzyme purified in this subfamily, CYP3A1 (Elshourbagy & Guzelian, 1980), was later shown to be induced by a wide variety of compounds, including endogenous and synthetic steroids (Schuetz & Guzelian, 1984; Simmons et al., 1987) and PB-like compounds (Schuetz et al., 1986). Expression of *CYP3A1* is totally dependent on the presence of an inducer, since no mRNA is detectable in untreated rats. In contrast, *CYP3A2*, an adult male-specific form isolated from rat (Guengerich et al., 1982), is constitutively expressed in adult male rat and it is only transiently expressed in young female rats. Moreover, expression of this gene is not induced by PCN, whereas both CYP3A1 and CYP3A2 mRNAs levels are elevated by phenobarbital. Hence, distinct and overlapping regulatory mechanisms play a part in the expression of these genes. The induction by glucocorticoids of the *CYP3A1* gene was shown to be due to increased transcription rates (Simmons et al., 1987). It is unlikely that the increase in transcription is mediated via a classical glucocorticoid response mechanism, since PCN was shown to inhibit induction of the rat Tyrosine Aminotranferase gene (TAT) by glucocorticoids (Schuetz & Guzelian, 1984). Interestingly, structure-activity relationships have been established for PB-like inducers that can induce also *CYP3A*

genes. Because no structure-activity relationship could be established among phenobarbital-like inducers when stimulating the expression of *CYP2B* genes, this would suggest that the components that regulate phenobarbital-mediated induction of *CYP3A* differ from those involved in *CYP2B* induction.

Macrolide antibiotics, such as triacetyloleandomycin (TAO), have also been shown to increase expression of CYP3A enzymes via post-transcriptional mechanisms (Dalet et al., 1988).

CYP3A enzymes catalyse the hydroxylation of certain steroids (testosterone 6-β- and 17α estradiol 2- and 4-hydroxylation), and the metabolism of some of its inducers, including TAO and erythromycin. It has also been reported that CYP3A3 is the major cyclosporine-metabolising enzyme in human liver (Kronbach et al., 1988).

CYP4 genes are regulated by hipolipidaemic drugs such as **chlofibrate** (CLOF) and other chemicals that also cause peroxisome proliferation and induction of peroxisomal enzymes (Kimura et al., 1989). These enzymes have a well-defined endogenous role in the metabolism of fatty acids, and arachidonic acid derivatives, such as prostaglandins and leukotrienes (Bains et al., 1985). The best-characterized reaction is the ω-hydroxylation of lauric acid. Clofibrate administration results in a rapid increase in the transcription rate of *CYP4A* genes (Hardwick et al., 1987). Recently, a peroxisome proliferator receptor (PPAR) was isolated, cloned and sequenced (Issemann & Green, 1990). This receptor was found to be a member of the steroid receptor superfamily, and it is characterized by having a ligand binding domain, and a DNA binding domain capable of interacting specifically with DNA sequences located upstream of *CYP4* genes. The ligand binding domain is equally capable of interacting with clofibrate and with endogenous lipids. It is thought therefore that this receptor, together with CYP4 enzymes, may be involved in lipid homeostasis. Indeed, it is as yet unclear whether clofibrate acts via direct binding to the PPAR, or acts indirectly by altering lipid homeostasis (Green, 1992). A combination of these two mechanisms may take place.

Certain rodent cytochromes P450 exhibit **sex-specific** expression. A central role for growth hormone and its secretion patterns has been shown in rats and mice, and a neuro-hepatic-endocrine axis that controls the regulation of some P450s has been identified (Waxman, 1988). In other species, including man, sex differences in drug metabolism are less pronounced (Skett, 1988).

II) P450 reductase

P450 reductase is a flavin-containing enzyme (Iyanagi & Mason, 1973; Vermillon & Coon, 1974). It is unusual in the fact that it has one mole of flavin adenine dinucleotide (FAD) and one mole of flavin mononucleotide (FMN), as non covalently bound co-factors, per mole of apoprotein (the chemical structures of FMN and FAD are shown in Fig. 1.12). Most other flavoproteins have either FAD or FMN as their prosthetic group. The enzyme has a monomeric molecular weight of approximately 78,000 Da and it is found in close association with cytochrome P450 in the endoplasmic reticulum (Fig. 1.2). This protein mediates the transfer of reducing equivalents from NADPH to cytochrome P450 and the reaction can be schematically represented as follows:

$$\text{NADPH} + \text{H}^{+} \longrightarrow (\text{FAD} \xrightarrow[\text{reductase}]{\text{P450}} \text{FMN}) \longrightarrow \text{Cytochrome P450}$$

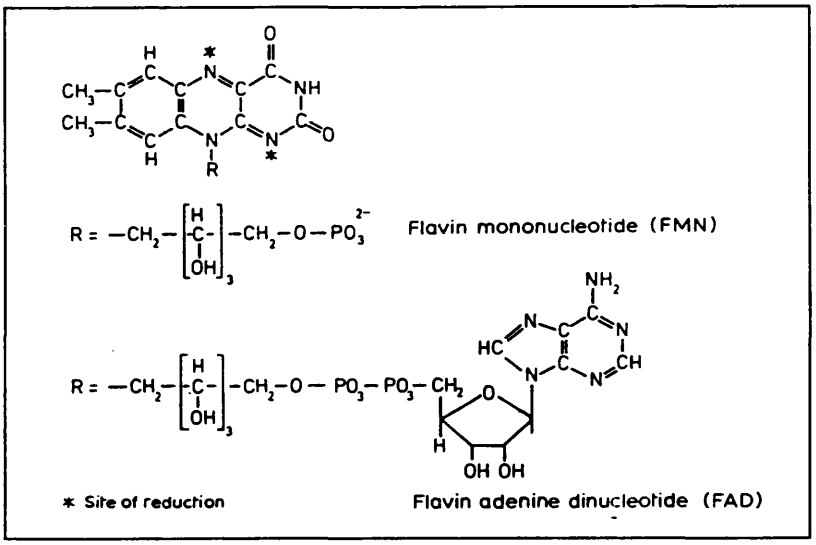


Fig. 1.12 Chemical structures of FMN and FAD

An intermediary electron transfer flavoprotein such as P450 reductase is necessary in the mixed function oxidase system because NADPH + H^+ is a two-electron donor whereas cytochrome P450 is a 2 x 1 electron acceptor. During the catalytic cycle, P450 reductase accepts two electrons from NADPH + H^+ and transfers them, one at a time, to cytochrome P450 (Lu et al., 1969). It is thought that FAD receives electrons from NADPH and FMN donates electrons to P450, however this has not been confirmed experimentally (reviewed in White, 1994). Both the cDNA and genomic DNA encoding P450 reductase have been isolated from several sources (Gonzalez & Kasper, 1982; Haniu et al., 1986; Katagiri et al., 1986; Urenjak et al., 1987; Yabusaki et al., 1988; Yamano et al., 1989; Porter et al., 1990). The rat gene is the best studied and consists of fifteen exons and is over 20 kb long (Porter et al., 1990). Several regions of the protein are conserved among flavoproteins and have been characterized as the FAD, FMN, and NADPH binding sites, and a membrane anchor sequence for insertion into the endoplasmic reticulum membrane. A region that interacts with cytochrome P450 has also been defined. The P450 reductase gene is coordinately regulated with several P450 genes. Administration of phenobarbital and PB-like compounds results in a 3-4-fold

transcriptional activation of the reductase gene (Hardwick et al., 1983).

III) Cytochrome b_5 and b_5 reductase

Cytochrome b_5 (relative molecular mass ≈ 18,000) and b_5 reductase (relative molecular mass ≈ 34,000) have both been isolated from the microsomal membranes of rat liver (Strittmater et al., 1978; Schafer & Hultquist, 1980). Cytochrome b_5 can be reduced by electrons transferred from NADH via the flavoprotein b_5 reductase or from NADPH via P450 reductase. It is well established (Oshino & Sato, 1971) that cytochrome b_5 is a necessary component of an electron transfer sequence required for one type of oxidative metabolism of fatty acids (Estabrook, 1978). However, the precise role of cytochrome b_5 in cytochrome P450 catalysed drug oxidations remains the subject of much controversy.

IV) Lipids

Early studies have shown the requirement for a lipid component in order to reconstitute mixed function oxidase activity (Haugen et al., 1976). The component was initially identified as phosphatidylcholine and later studies showed the fatty acid composition of the phospholipid to be critical (Nelson & Strobel, 1988). The exact mode of action of lipids is as yet unknown but it has been suggested that lipids may be required for substrate binding, facilitate electron transfer or provide a template for the correct interaction between cytochrome P450 and P450 reductase (reviewed in Black, 1992; Sakaguguchi & Omura, 1993). Indeed, it was shown that only the intact membrane binding form of the reductase can reduce cytochrome P450s, even if a soluble, trypsinized form can be obtain that can reduce cytochrome c (Black & Coon, 1986).

1.2.2 Microsomal Flavin Containing Monooxygenases

Flavin containing monooxygenases (FMO; reviewed in Ziegler, 1991, 1993; Lawton & Philpot, 1993) are a group of microsomal enzymes involved in the oxidative metabolism of a variety of xenobiotics that possess so-called "soft nucleophilic atoms" (i.e. nitrogen, sulphur, selenium and phosphorus). Consequently, FMO substrates include numerous pesticides, drugs, pollutants, and plant extracts. Endogenous compounds have not yet been identified and the physiological role of these enzymes is still unclear. The FMO enzyme is a polymeric protein exhibiting a monomeric molecular weight that ranges between 56,000 and 63,000 (Ziegler & Mitchell, 1972; Dannan & Guengerich, 1982, Kimura et al., 1983; Sabourin et al., 1984; Dolphin et al. 1991, 1992) and containing one mole of flavin adenine dinucleotide (FAD) per protein monomer (Smyser & Hodgson, 1985). FMOs have been found in many tissues, with highest concentrations being found in the microsomal fractions of liver and lung and they are present in substantial amounts in man and hog and, in smaller amounts, in smaller mammals such as rats (Dannan & Guengerich, 1982). Prokaryotic forms of this enzyme have also been identified (Ryerson et al., 1982; Agosin & Ankley, 1987). Examples of general reactions catalysed by this enzyme are given in Fig. 1.13. Using *N, N*-dimethyl-aniline as a representative substrate, the reaction catalysed by FMO has the following stoichiometry:

$$PhN(CH_3)_2 + O_2 + NADPH + H^+ \xrightarrow{FMO} PhN^+O^-(CH_3)_2 + NADP^+ + H_2O$$

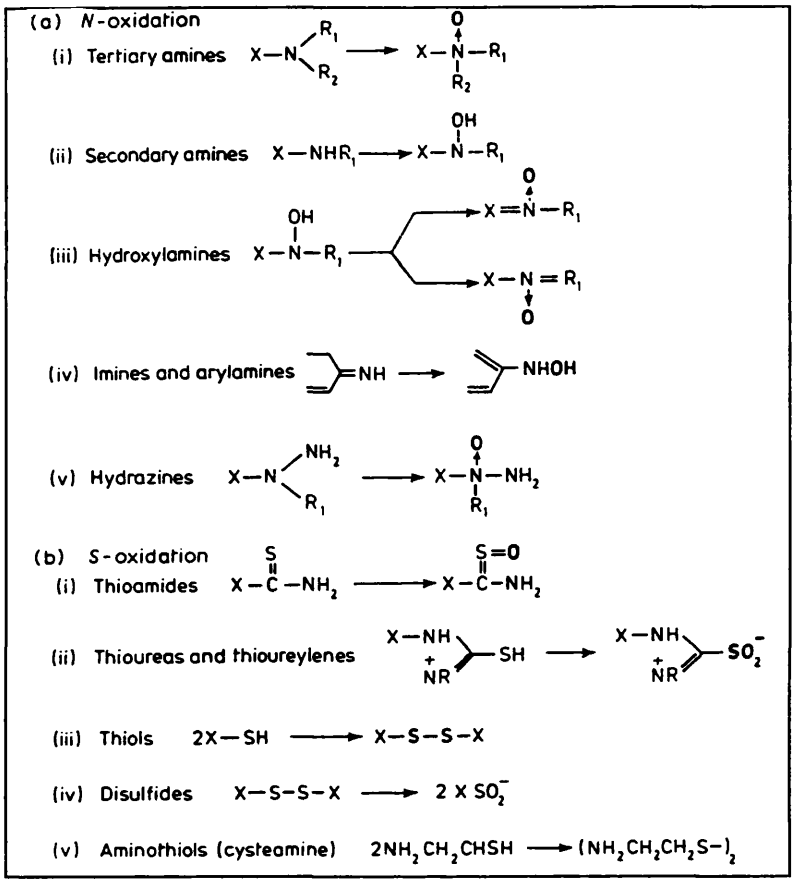


Fig. 1.13 Examples of general reactions catalysed by FMO. Reproduced from Gibson & Skett (1994)

The catalytic cycle of the FMO reaction is fairly well understood (Ziegler, 1988; Fig. 1.14). The flavin monooxygenases can accept reducing equivalents from either NADH or NADPH. However, NADPH is the preferred cofactor since its K_m is ten times lower than that for NADH. Unlike all other monooxygenases bearing flavin or other prosthetic group, substrate binding is not required for dioxygen reduction by NADPH (Ziegler, 1991). Instead, flavin reduction (step 1 of catalytic cycle depicted in Fig. 1.14), oxygen binding (step 2), and internal transfer of electrons to oxygen forming the peroxy-flavin complex (step 3) can take place spontaneously, in the absence of a substrate. Indeed, in cells the majority of the enzyme appears to be present in this highly reactive 4a-hydroperoxyflavin form, which the surrounding protein microenvironment is thought

to stabilise. Any soft nucleophile that can make contact with this very potent monooxygenating agent will be oxidised (Ball & Bruice, 1986). The product, formed by oxygen transfer from the hydroperoxyflavin to the nucleophile is released immediately (steps 4 and 5). This mechanism confers the very broad substrate specificity characteristically associated with FMO enzymes. The remaining step of the catalytic cycle will simply restore the enzyme to its oxidised form. In the absence of a suitable substrate, the peroxy-flavin intermediate will slowly decompose with the concomitant release of hydrogen peroxide (step 7).

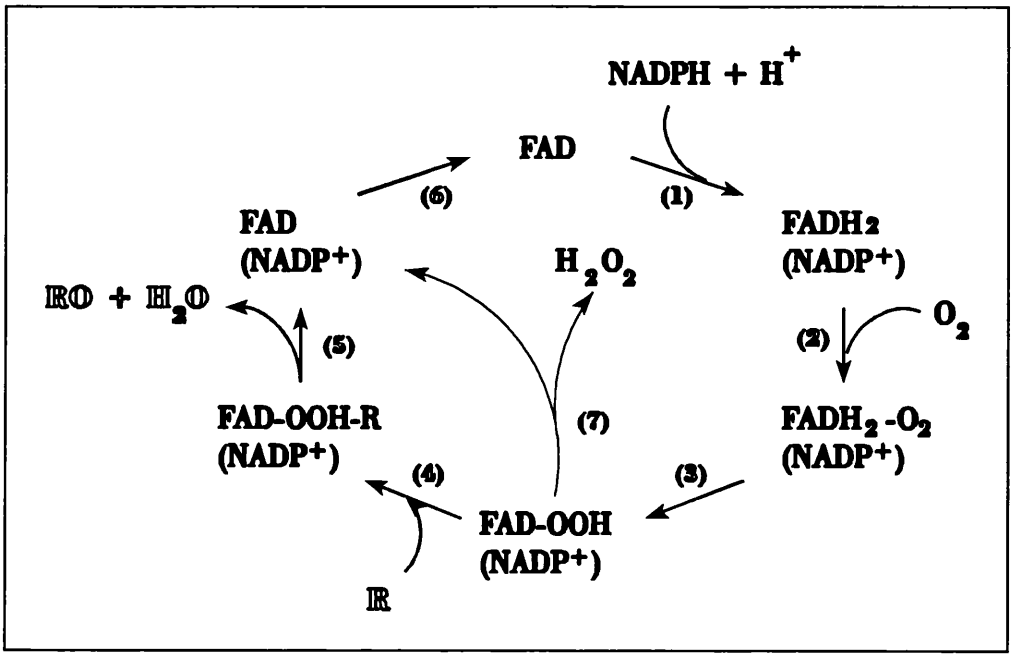


Fig. 1.14 Catalytic cycle of FMO reaction. "R" : substrate. Reproduced from Gibson & Skett (1994).

The question as to the number of different forms of FMO that may exist and their tissue-specific expression is a topic of active research. It was initially thought that only one form of FMO existed in mammalian tissues (Machinist et al., 1968; Ziegler & Mitchell, 1972). However, to date at least five different mammalian cDNAs have been isolated and sequenced (Gasser et al., 1990; Lawton et al., 1990; Ozols, 1990; Dolphin et al., 1991, 1992; Ozols, 1991; Lomri et al., 1992; Shephard et al., 1993). A nomenclature system based on comparisons of amino acid sequences has been recently proposed (Lawton et al., 1994). In this system, the mammalian flavin-containing monooxygenase gene family is designated as "FMO" and individual genes are distinguished by an Arabic numeral. The genes and cDNA designations will be italicized and the mRNA and protein designations will be non-italicised. Hence, *FMO1*, *FMO2*, ...*FMO5* will indicate the genes encoding FMO1, FMO2, ...FMO5 mRNAs and proteins, respectively. The order of naming follows the chronology of publication of full-length sequences.

In addition to their intrinsic interest, FMOs are of clinical relevance since polymorphisms associated with one or more of these genes (Al Waiz et al., 1987) are the cause of a human pathological condition known as trimethylaminuria or Fish Odour Syndrome (FSO) (Humbert et al., 1970; Shelley & Shelly, 1984). Subjects affected by this condition are poor metabolizers of trimethylamine (TMA) (Al Waiz et al., 1987b), a highly volatile, odorous compound that is responsible for the characteristic putrid smell of rotten fish. FMO enzymes catalyse the conversion of TMA into the N-oxide equivalent, a non-odorous, non-volatile compound (Hlavica & Kehl, 1977). Lack of, or reduced catalytic activity towards TMA causes the affected person to produce a peculiar fish-like odour, with the consequence that patients suffer from social exclusion. The incidence of suicide among patients is extremely high, and because this syndrome does not lead to any physical abnormality other than the smell, it is often ignored and/or misdiagnosed. Therapeutic protocols at the moment are mainly focused around carefully

prepared diets that lack TMA, however a more detailed understanding of the molecular biology and the genetics of these genes would be very beneficial.

FMO genes have been shown to be under tissue-, sex-, species and substrate-dependent regulation by hormones (Skett, 1987; Lemoin et al., 1991; Lee et al., 1993). Interpretations of the results arising from studies on the modulation by xenobiotics of FMO expression *in vivo* have been made difficult by the fact that commonly used commercial rat chow has been shown to contain substantial amounts of xenobiotic soft nucleophiles (Ziegler, 1993). A rapid drop in total FMO protein and activity was observed when rats were kept on chemically-defined diets for as little as two days (Kaderlik et al., 1991). These results seem to suggest that FMOs and other drug metabolising enzymes that are regulated by soft nucleophile xenobiotics may already be maximally expressed in rats fed on commercial chow, and that for this reason the early negative results obtained when trying to induce FMO expression *in vivo*, by phenobarbital or 3-MC administration may be inconclusive (Masters & Ziegler, 1971). Little is known about FMO expression in cultured cells, however, due to the fact that chemically-defined culture media can be employed, cell cultures could represent a useful tool for the study of xenobiotic induction of these enzymes.

1.2.3 Phase II drug metabolising enzymes

As mentioned in section 1.1, xenobiotics activated by Phase I enzymes are subjected to metabolic conjugation (and other kinds of detoxification) by Phase II enzymes, leading to ultimate excretion of the hydrophilic (water-soluble) metabolites into the urine or bile. Most of the conjugation reactions are catalysed by a variety of transferases, including sulfotransferases, UDP-glucuronosyl transferases, and glutathione transferases.

The **sulfotransferases** (reviewed in Falany, 1991) catalyse the transfer of sulphate from adenosine 3'-phosphoadenylylsulfate to a variety of phenolic compounds and primary and secondary alcohols which include primary and secondary hydroxysteroids. They are soluble enzymes found in a variety tissues, including liver, kidney, gut, and platelets. They are of some clinical interest because they are involved in the metabolism of drugs like paracetamol, isoprenaline and salicyl-amide. However, due to the limited bioavailabilty of sulphur (which is converted to the activated form adenosine 3'-phosphoadenylylsulfate), sulfation reactions are not as widespread as other conjugation reactions.

UDP-glucuronosyl transferases (reviewed in Tephly & Burchell, 1990) catalyse the transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to xenobiotics or endobiotics for glucuronide formation. They are found in almost all mammalian species and are present in a variety of tissues, mostly in the liver, but also in the kidney, small intestine, lung, skin, adrenals and spleen. The enzyme is mainly localized in the membranes of hepatic endoplasmic reticulum and therefore is ideally placed to glucuronate the products of phase I reactions.

Glutathione conjugation is of particular importance in being one of the major defence mechanisms in the body against xenobiotic electrophiles (many of which are mutagens and/or carcinogens).

1.2.3.1 Glutathione S-transferases: general background and nomenclature

The glutathione S-transferases (GST) are a family of enzymes that catalyse the nucleophilic attack of the sulphur atom of glutathione (Glu-Cys-Gly) on the electrophilic centre of a variety of chemical compounds (reviewed in Mannervik & Danielson, 1988). These enzymes are predominantly cytosolic proteins, however a microsomal form of

GST has been identified in rat (Morgenstern et al., 1985) and man (McLellan et al., 1989). They catalyse the following general reaction, where R-CH_2 -X represents literally hundreds of electrophilic substrates and R-CH_2 -SG represents the glutathione adduct:

$$\text{R-CH}_2\text{-X} \xrightarrow[\text{glutathione S-transferase}]{\text{GSH}} \text{R-CH}_2\text{-SG}$$

In addition to the conjugation reaction shown above, certain GSTs have GSH-independent peroxidase activity (Tan et al., 1988; Meyer et al., 1991), allowing these enzymes to play an important role in protecting tissues from the negative effects of endogenous hydroperoxides, produced as an effect of oxidative stress. Some GSTs are also capable of binding, without metabolism, to a variety of endogenous and exogenous substrates, such as leukotriene C_4, bilirubin, steroid and thyroid hormones, and a number of drugs and carcinogens (Boyer, 1989; Ishigaki et al., 1989; Vos & Van Bladeren, 1990). This suggests that they may have a role also as intracellular carrier proteins.

Mammalian GSTs have been thoroughly investigated. These studies have shown that cytosolic glutathione S-transferase enzymes exist in multiple forms as homo- or hetero-dimers in almost all tissues investigated. The monomeric molecular weight of these proteins ranges between 21,000 and 29,000 (reviewed in Mannervik, 1985; Mannervik & Danielson, 1988). In contrast, the microsomal enzyme exists as a trimer (Morgenstern et al., 1988). Nomenclature of GST enzymes has been traditionally confusing as several different systems have been employed. Based on sequence homology, and immunological cross-reaction, the cytosolic forms have been classified into four multigene classes, namely α, μ, π, and θ (Meyer et al., 1991b). However, the membrane bound, microsomal form is not a member of the above four classes and it appears to have a distinct evolutionary origin from the cytosolic enzymes (Fig. 1.15 and Table 1.2). The nomenclature system proposed by Jakoby et al. (1984) is often used for

rat and mouse GSTs. In this system, GST subunits are identified by an Arabic numeral assigned according to the chronology of isolation and characterisation. Recently (Mannervik et al., 1992), a unifying nomenclature for the human glutathione transferases has been suggested, primarily based on the four major classes of soluble enzymes (Alpha, Mu, Pi, Theta) and reflecting their subunit compositions. Thus, GSTA1-1 is a glutathione S-transferase (GST) of the Alpha class, consisting of a homo-dimer of two "1"-type subunits. However, this nomenclature has not yet been applied to glutathione from other organisms.

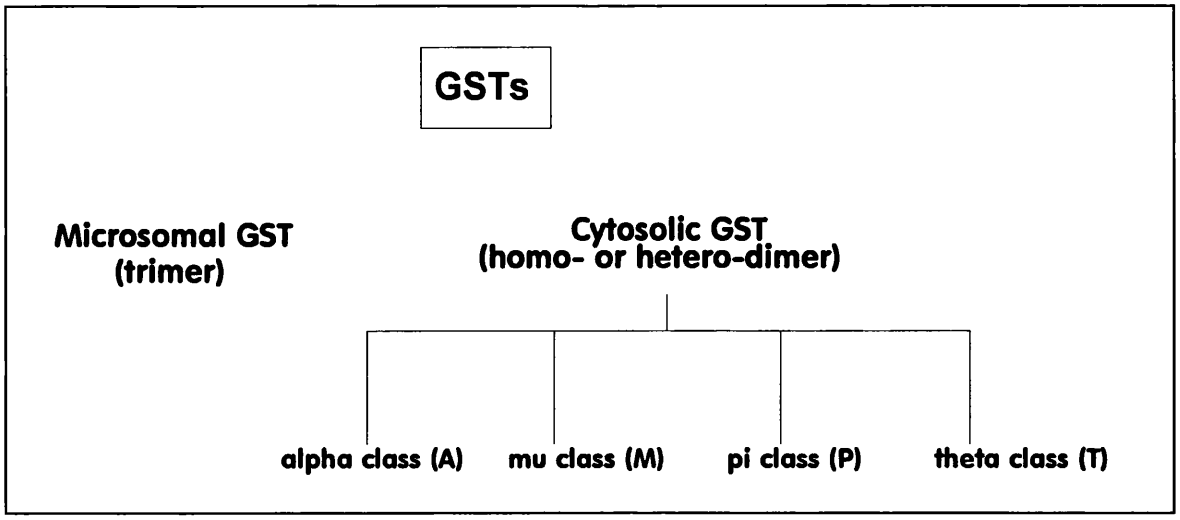


Fig. 1.15 GST multigene family.

Table 1.2 Classification of GST subunits into different classes (rat and mouse)

Class	Sub-unit	GST name
Alpha	1a, 1b	GST Ya
	2	GST Yc
	8	GST Yk
	10	
Mu	3	GST Yb1
	4	GST Yb2
	6	
	9	
Pi	7	GST Yp
Theta	5	
	12	
(mitochondrion)	13	

Most GSTs can catalyse the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), which is regarded as a "universal" substrate (reviewed in Mannervik & Danielson, 1988). As a group, GSTs display overlapping substrate specificities. However, each class of subunit has a characteristic substrate specificity profile. For example, alpha class GSTs are highly active against organic hydroperoxides such as cumene hydroperoxide (Sexton et al., 1993); mu class GSTs show highest activities with most epoxides (Mannervik & Danielson, 1988); pi class GSTs have comparatively high activity against ethacrinic acid (Van Bladeren et al., 1993); theta class GSTs are mainly active against 1-menaphtyl sulphate (Meyer et al., 1991). Microsomal GSTs catalyse the conjugation of GSH with hexachlorobuta-1,3-diene (Morgenstern et al., 1988).

1.2.3.2 Glutathione S-transferases: gene structure and regulation

Glutathione S-transferase genes have been shown to be induced, mainly at the level of transcription, by a large number of compounds, including phenobarbital and polycyclic aromatic hydrocarbons (reviewed in Daniel, 1993). The study of the mechanisms involved in GST induction have been hampered by the fact that GST expression is either extinguished or altered when hepatocytes are placed in culture. Differential changes, during the culture period, can be summarised as a general loss of the α class of subunits, a maintenance or increase in the μ class of subunits, and the *de novo* expression of subunit 7 (Vandenberghe et al., 1988, 1992). This pattern of expression of GST enzymes (subunit 7 expression in particular) is indicative of a general state of dedifferentiation. Two differentiated cell lines, FAO and H4II, that still express a large number of liver-specific functions (Deschatrette & Weiss, 1974) have often been employed. In these cells it was possible to show, by RNA dot hybridization, the induction of GST 1 mRNA by phenobarbital and 3-MC (Daniel et al., 1989). However, in other cases it has been necessary to monitor the activity NAD(P)H:quinone reductase, an enzyme activity that is

co-ordinately induced with GST in animal tissues and is still expressed in a number of hepatoma cells (De Long et al., 1987). From these experiments and others, a model was proposed that implies a unified mechanism for induction of Phase II enzymes by all classes of chemical compounds. According to this model the induction ability of a chemical compound is dependent on its electrophilicity and its ability to generate a redox signal (i. e. oxidative stress). In the case of inducers like phenobarbital or TCDD (classed as bifunctional inducers because they are capable of inducing both Phase I and Phase II enzymes), induction of Phase II enzymes would be mediated by an increased metabolic activity of cytochromes P450. This, in turn would lead to an increase in the number of electrophiles present in the cell (i.e. oxidative stress). Mono-functional inducers (those that specifically induce Phase II enzymes) are already reactive electrophilic molecules and capable of generating a redox signal. This model however does not exclude that compounds like TCDD may increase GST transcription also via the Ah receptor, provided the necessary regulatory elements can be found in GST promoters. Most of what is known about GST gene expression comes from studies on the rat and mouse subunit 1 gene, which belongs to the alpha gene family.

A) GST alpha gene family

cDNA clones complementary to the mRNAs encoding GST 1 and 2 subunits have been isolated from a variety of tissues of rat, mouse, and other mammals, including man (reviewed in Daniel, 1993). GST 1 and 2 proteins have a molecular weight of ≈26,000 and at least 75 % of their amino acids is identical. However, the 5' and 3' untranslated regions of the mRNAs encoding these two proteins are very divergent (Pickett & Lu, 1989). DNA sequence analysis of different rat liver GST 1 cDNAs has revealed the existence of two highly homologous GST 1 subunits, named 1a and 1b, which differ by only eight amino acids (Lai et al., 1984; Pickett et al., 1984; Taylor et al., 1984). The structural gene encoding the GST 1 subunit has been isolated from rat (Telakowski-

Hopkins et al., 1986), and mouse (Daniel et al., 1987). Both genes have seven exons and are 11 kb in length. DNA sequences flanking the 5'-end of both genes have also been isolated and characterised (Telakowski-Hopkins et al., 1988; Daniel et al., 1989). Increased transcription of the mouse GST 1 gene, as a function of oxidative stress, appears to be mediated by a *cis*-acting regulatory element, located between -754 and -714 of the 5'-flanking sequence, that has been referred to as **electrophile-responsive element** (EpRE) (Friling et al., 1990). A sequence very similar to that of EpRE was also found in the 5'-flanking sequence of the rat GST 1 gene (Rushmore et al., 1990). Because of its activation by a phenolic antioxidant, this regulatory element was called **antioxidant-responsive element** (ARE). The EpRE was found to contain a 9 bp direct repeat, spaced by six bp, that are a direct variant of the consensus for Activator Protein-1 (AP-1). This regulatory element was originally identified as the phorbol 12-myristate 13-acetate (PMA) responsive element and it was shown that it interacted with the protein products of *c-fos* and *c-jun* genes (Angel et al., 1987). The preferential hepatic expression of alpha class GST may be mediated by two liver-enriched transcription factors, HNF-1 and HNF-4 whose potential binding sites were identified upstream of the rat GST 1 gene (Paulson et al., 1990).

B) GST Mu gene family

The cDNA clones of subunit 3, 4, and 6 have been isolated and characterized (Ding et al., 1985, 1986; Lai et al., 1987). A sequence comparison among these clones shows that they share 80 % amino acid sequence identity. The genes coding for subunits 3, 4, and Yb_4 have been isolated (Lai et al., 1988; Morton et al., 1990). The Yb_4 gene codes for a putative Yb_4 subunit that has not yet been purified from any rat tissue, and there is no evidence that it may be expressed. The gene coding for subunit 6 has not yet been isolated , but using cDNA as a probe, it has been shown to be expressed mainly in the brain, and very little in liver (Abramovitz & Listowski, 1987). The structures of the

Yb genes isolated (reviewed in Taylor et al., 1991) is very similar: they are approximately 5 kb in length and contain eight exons. Four human cDNAs have also been isolated and characterized, GSTM1a (De Jong et al., 1988b), GSTM1b (Seidegard et al., 1987), GSTM2 (Voraceck et al., 1991), and GSTM3 (Campbell et al., 1990). GSTM1a and GSTM1b only differ by a single base pair in the protein coding region, with a resulting change in amino acid residue 172 from Lys to Asn. GSTM2 was isolated from myoblasts and GSTM3-3 isoenzyme was found in brain and testes. About 50 % of the human population lacks GST catalytic activity towards *trans*-stilbene oxide (Board, 1981). This has been shown to be due to a deletion in the GSTM1 gene (Seidegard et al., 1988) and it has been correlated to a possible increase in lung cancer incidence among smokers (Nazar-Stewart et al., 1993).

C) GST Pi genes

cDNA clones encoding rat (Pemble et al., 1986) and human GST (Kano et al., 1987) pi subunits have been isolated, together with the corresponding structural genes (Okuda et al., 1987; Cowell et al., 1988). Both rat and human genes span about 3 kb, contain seven exons, and encode a protein 210 amino acids long. Unlike other classes of GST subunits, only one gene exists in this class, in both species. The GST pi class is of particular interest because its expression is associated with carcinogenesis (Sugioka et al., 1985). Placental GST-P (or GST 7-7), the only pi class gene present in rats, is absent from normal adult hepatocytes, and is specifically induced at an early stage of chemical hepatocarcinogenesis, and it appears to be a good marker for carcinogenesis in rat. The expression of the GST-P gene is developmentally regulated. It is expressed at high levels in foetal liver, but it rapidly decreases after the first week of post-natal development to become undetectable in adult liver (Abramovitz & Listowsky, 1988). As already mentioned, the expression of GST-P (GST 7, GST π) is markedly increased in cultures of primary hepatocytes. This has been shown to be due to increased transcription of the

corresponding gene (Vandenberghe et al., 1992) and phenobarbital was shown to greatly increase GST-P mRNA levels in culture (Vandenberghe et al., 1988, 1989). Analysis of the 5'-flanking sequence of the rat GST-P gene has revealed a number of *cis*-acting DNA regulatory elements. In particular, an AP-1 binding site and a GC box, located at positions -61 and -40 respectively, have been shown to contribute to the basal level of GST-P gene expression (Sakai et al., 1988). A stretch of transcription factors binding sites, stretching between -396 to -140, were shown to constitute a silencer element (Imagawa et al., 1991). At least three transcription factors were found to bind to this region, and one of them was identified as the liver-enriched C/EBPβ transcription factor (Imagawa et al., 1991). Two enhancer elements were also identified at -2.2 kb, and at -2.5 kb, respectively named GPEII and GPEI (Okuda et al., 1989). GPEI was shown to be similar in structure to EpRE, and to contain two adjacent AP-1 binding sites. Interestingly, the 5'-flanking region of the human equivalent of the GST-P gene, GSTP (GST-π), lacks this enhancer, and this may account, at least in part, for the differences observed in its pattern of expression. GSTP levels are increased in human malignant disease and in some human tumour cell lines that have acquired resistance to antitumour drugs. GSTP, however, is not expressed in hepatocellullar carcinoma and, unlike its rat counterpart, does not appear to be induced in cells that are characterized by elevated levels of oncogenes (*ras*) expression or exposed to PMA (Dixon et al., 1989).

D) Theta class GST gene

Two theta class GST genes have been found in human liver and partial analysis of their amino acid sequence shows that they are related to the three rat and one dog theta GSTs so far identified (Hiratsuka et al., 1990; Meyer et al., 1991b). Neither of the human GST genes have been cloned, however two rat cDNAs were shown to code for two peptides of about 240 amino acids in length, with an approximate molecular weight of 27,000.

E) Microsomal GST gene

The cDNA clone of human microsomal GST encodes a 154 polypeptide with a molecular weight of about 17000 Da. It shares 83 % amino acid similarity with its rat ortholog but has only limited sequence similarity to cytosolic GSTs. (De Jong et al., 1988)

1.3 *In vitro* studies of drug metabolism and scope of this thesis

I hope, by the information provided in the previous paragraphs, that I have managed to convey two major aspects of the study of drug metabolism:

1. Because of the complexity of Phase I and Phase II interactions, drug metabolism studies involving whole organisms are difficult to interpret, since carefully controlled experimental conditions are difficult to achieve. For this reason, statistical evaluations involving large sample groups are often required, which, taking into consideration the high costs for the care and maintenance of experimental animals, render *in vivo* drug metabolism studies very expensive. Tissue homogenates (microsomes and cytosolic cell fractions) have been often employed as a relatively simple, low-cost alternative. This methodology has proven its value especially in studies of specific biotransformation steps, e.g. immunoinhibition of specific cytochrome P450 enzymes, or for the detection and production of reactive intermediates. Indeed, for this type of study they are still the method of choice. However, subcellular fractions lack the presence of an intact intracellular compartment, where appropriate cellular uptake processes, intracellular distribution of chemical compounds, cofactor supply and the arrangement of several biotransformation enzyme systems can take place. For this reason, *in vitro* biotransformation studies that employ intact isolated liver cells are potentially the ideal

compromise. Indeed, drug metabolism induction is a function of intact cells and does not occur in tissue homogenates. Isolated liver cells represent responsive entities and can therefore be utilised for regulation studies. It should be emphasized at this point that, for a final assessment, *in vitro* alternatives cannot generally replace whole-animal experiments. They only represent a model system from which indications for subsequent *in vivo* research can be drawn. In fact, to a certain extent, and especially in drug metabolism studies where inter-individual variations are so evident, even *in vivo* studies on one individual organism can only amount to a model system for another individual.

2. Intact isolated liver cells can be employed either in suspension or in culture. Cells in culture offer the obvious advantage that they can be employed for long term studies, a prerequisite for studies on the regulation of drug-metabolising enzymes (Guillouzo, 1986). In addition, hepatocytes are given time to recover from alterations sustained during the isolation procedure, and can be allowed to acclimatize to a carefully controlled chemical environment. However, a serious limitation to the use of isolated liver cells has been the rapid decrease in drug metabolising enzymes, particularly some cytochromes P450 and some glutathione S-transferases, following their isolation and placing in culture. Hepatoma cell lines were also found to lack cytochrome P450 expression.

Much effort has been devoted to try to find culture conditions that could restore the expression of these enzymes to levels that mimicked those observed *in vivo.* It is worth mentioning at this point that it is difficult to define what the " *in vivo"* levels of expression should be. Since experimental animals are continuously exposed to xenobiotics, most of which are inducers of drug metabolising enzymes, the overall pattern of P450 expression observed *in vivo* at any given time may be the co-ordinated effect of a variety of factors that are not going to be present in the chemically defined conditions of cell culture. It is possible therefore that the observed reduction in P450 levels may be in part due simply to the removal of inducing factors.

Generally speaking, reduction in P450 expression appears to be due to a general foetalization that isolated hepatocytes undergo when cultured under "standard" conditions. It is possible that this is due to a gradual loss of liver-enriched transcription factors, leading to dedifferentiation. As mentioned in previous paragraphs, the induction of *CYP2B* genes by phenobarbital has been particularly difficult to achieve in culture and this inductive response appears to be a very good marker for hepatic differentiation. The absence of a responsive cell culture system (hepatoma cell lines tested are generally refractive to PB induction) has also greatly hampered mechanistic studies in the regulation of the transcriptional activation of these genes. Many investigators therefore have dedicated much work in attempts to define culture conditions that could reproduce PB induction of *CYP2B* genes in culture. Empirical studies have led to the identification of culture parameters that can influence the stability of hepatocytes and/or their maintenance of liver-specific functions in culture. The extracellular matrix, culture medium, the presence of added hormones, cell density, cell-cell and cell-matrix interactions have all been shown to affect the phenotype of cultured cells (reviewed in Guillouzo, 1986; Waxman et al., 1990; hormone/matrix synergy and liver differentiation are reviewed in Reid, 1990). Several recent studies have established a reproducible method whereby primary rat hepatocytes cultured on a substratum of Matrigel (a reconstituted extracellular basement membrane extracted from the Englebreth-Holm-Sarcoma), maintain their PB response as well as a number of other differentiated functions (Ben-Ze'ev et al., 1988; Schuetz et al., 1988). However, the requirement of a Matrigel substratum for maintenance of a PB-response was disputed in two subsequent reports (Sinclair et al., 1990; Waxman et al., 1990), which demonstrated that a similar induction response, at least at enzyme activity levels, could be achieved by manipulating the culture medium. In both cases, however, culturing cells on an extracellular matrix appeared to be advantageous. In particular, in the latter report, a method was employed to covalently bind collagen to the plastic of culture dishes characterized by their

particularly soft plastic (Permanox®). This achieved an improved coating of the dishes that, together with the use of a highly enriched, chemically defined medium (Dr. Chee's modification of Eagle's medium), achieved long-term preservation of cultured cells and demonstrated increased CYP2B related activities as a response to PB. This study also showed that growth hormone was inhibitory to PB induction in culture. As the use of Matrigel appears to be inhibitory to current techniques for transfection of DNA into primary hepatocytes (Pasco & Fagan, 1989), the culture conditions of Waxman et al. (1990) appeared promising. A recent report (Sidhu et al., 1993), however, has shown that the increases in CYP2B related activities, as a function of PB, were due to mRNA stabilization, rather than to an increase in mRNA levels. In addition, this study was in agreement with previous reports (Sinclair et al., 1990; Waxman et al., 1990), showing that induction was not exclusively dependent on the presence of Matrigel. However, they observed that PB induction was strongly augmented if cells were plated on Vitrogen (type I collagen)-coated plates, and then overlaid with a dilute Matrigel solution. They also confirmed previous findings (Sinclair et al., 1990) that the PB induction is superior in cells cultured with William's E, than in Chee's or Waymouth's media.

The requirement for cell-cell interactions in maintaining adult liver functions has been demonstrated by co-culturing primary hepatocytes with biliary epithelial cells (Akrawi et al., 1993). This culture system has been shown to support high levels of PB induction of CYP2B genes and co-cultured cells express many of the functions of adult rat hepatocytes. However, the presence of a second cell-type in culture can be a complicating factor when interpreting results and can render studies on the regulation of gene expression difficult to perform. Moreover, the isolation of biliary epithelial cells is technically demanding.

The aim of my project was to develop relatively simple culture conditions that could support the expression of drug metabolising enzymes and could be employed in studies designed to investigate the molecular mechanisms involved in PB induction of

CYP2B genes. Much effort in our laboratory is devoted to this aim and the knowledge acquired using other *in vitro* techniques could be selectively employed in a responsive cell culture system.

Chapter two

Materials and Methods

2.1 Chemicals

All chemicals, except those used for tissue culture and unless otherwise specified, were analytical reagents grade and were purchased from BDH, UK. Sodium phenobarbital and picrotoxin were purchased from Sigma Chemical Co., UK. Chemicals used for tissue culture were tissue culture grade. William's E medium, Chee's Modified Eagles' Medium (CMEM), Earle's Balanced Salt Solution (EBSS; 10 x, without calcium and magnesium), Eagle's modified Minimal Essential Medium (EMEM; with Hanks' salts), amphotericin B, and neomycin were puchased from Gibco BRL, UK. Streptomycin and penicillin were purchased from Sigma Chemical Co., UK. Collagenase H was purchased from Boehringer Mannheim Ltd., UK. Vitrogen 100® was purchased from Imperial Laboratories, UK.

2.2 Primary hepatocyte isolation and culture

2.2.1 Hepatocyte isolation

Primary hepatocytes were isolated from the livers of adult, male Sprague-Dawley rats weighing 200-250 g by a modification of the two-step collagenase perfusion of Seglen (1976). Prior to surgery, animals were anaesthetised by i.p. injection (100 µl/ 100 g of body weight) with a sterile 60 mg/ml solution of Sodium Pentobarbital (Sigma Chemical Co., UK) in 0.9 % NaCl. When the animals were fully unconscious, the abdomen was shaved and cleaned with a 70 % solution of ethanol. Using blunt-end scissors, the abdomen was cut open and the intestinal pack gently shifted to the right hand side, in order to reveal the *vena porta*. Two loose ligatures were placed around this vein using a *Deschamps* ligature needle (Lawton Gmb.H. & Co., Tuttlingen, Germany) and *Mersilk Black 3* ligature thread (Ethicon Ltd., UK). A third loose ligature was placed around the *vena subrenalis*. At this point, in order to prevent the formation of blood clots that could inhibit perfusion of the liver, the rat was injected i.v. with 1 ml of a 200 U/ml solution of heparin (Sigma Chemical Co., UK). After 20-30 seconds, the

bottom ligature surrounding the *vena porta* was tightened, and a flat cut was made in the vein between the tight and loose ligatures. A glass cannula, previously filled with perfusion buffer 1 [1 x EBSS, 0.2 % $NaHCO_3$ (w/v), 0.5 mM EGTA, 0.002 N NaOH, 100 μg/ml streptomycin, 100 U/ml penicillin, pH 7.5, 37° C] was inserted into the *vena porta* and held in place by tightening the top ligature around it and securing it with a double knot. At this point the ligature surrounding the *vena subrenalis* was also tightened. Making sure that no damage was inflicted to the *glissen* capsule, the liver was then gently removed using blunt-ended, curved *Mayo* scissors (Lawton Gmb.H. & Co., Tuttlingen, Germany), and transferred to the perfusion apparatus (Chapter 3, Fig. 3.1). The cannula was connected to the apparatus by means of Tygon® 50 dialysis tubing (Verder Belgium, Belgium), and perfusion commenced. The liver was perfused for two minutes with perfusion buffer 1, then for 10 minutes with perfusion buffer 2 [1 x EBSS, 0.2 % (w/v) $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, pH 7.5], and finally for 20-25 minutes with perfusion buffer 3 [1 x EBSS, 0.2 % (wt/vol) $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM $CaCl_2$, 0.08 U/ml Collagenase H, 0.001 % (w/v) soya bean trypsin inhibitor (Sigma Chemical Co., UK), pH 7.5]. During perfusion, buffers were gently gassed with 95 % air / 5 % CO_2, and constantly heated so that their temperature was 37° C when they reached the liver. After perfusion was complete, the liver was immediately transferred to a petri dish containing 40 ml of Leibovitz L-15 medium (Sigma Chemical Co., UK), supplemented with 2.5 % (w/v) bovine serum albumin (BSA; fraction V, Sigma Chemical Co., UK). The *glissen* capsule was gently removed with sterile forceps and the cells were dispersed into the medium by a gentle combing action. The cell suspension was then filtered though a sterile nylon mesh (62 μ; BDH, UK.) to remove large debris of connective tissue. Cells were then pelleted by centrigugation (50 x g, 2 min., 4° C). For a final wash, cell pellets were gently resuspended in 50 ml of dispersal buffer [10 mM HEPES, 142 mM NaCl, 7 mM KCl, 7 mM $MgSO_4$, 10 mM $CaCl_2$, 2.5 % (w/v) BSA (fraction V, Sigma Chemical

Co.)], and centrifuged for 2 min. at 50 x g (4° C). Cell pellets were then resuspended in complete media.

2.2.2 Hepatocyte culture

Cell pellets obtained after the final wash of the isolation procedure, were resuspended in William's E Medium supplemented with insulin (1.7 μM), dexamethasone (0.1 μM), streptomycin (100 μg/ml), penicillin (100 U/ml), amphotericin B (2.5 μg/ml), neomycin (200 μg/ml), L-glutamine (4 mM), and 5 % dialysed foetal calf serum (Gibco BRL, UK). Cells (4-5 x 10^6) were then plated onto Vitrogen-coated or uncoated Permanox® plates (60 mm; Nunc, UK) in a volume of 2 ml and left to attach for 3 hours at 37° C, in a humidified atmosphere of 95 % air and 5 % CO_2. Permanox plates were coated with Vitrogen using CDI (1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate), (Aldrich Chemicals UK) as the coupling agent, essentially as described in Waxman et al. (1990). Permanox plates were coated with 3 ml of a fresh solution of Vitrogen (90-100 μg/ml) and CDI (130 μg/ml) and incubated at 37° C overnight. Unbound collagen was then removed by aspiration and the plates washed twice with a sterile 0.9 % NaCl solution and stored at 4° C for up to a week until ready to use. Plates were washed once more with 0.9 % NaCl, just prior to use.

After attachment, the culture medium was aspirated and replaced with 7 ml of fresh William's E medium, containing all supplements except foetal calf serum. Cells were then incubated at 37° C in a humidified atmosphere of 95 % air and 5 % CO_2. Culture medium was changed at 24 hr intervals. In some initial experiments where we compared different culture conditions, isolated hepatocytes were also cultured in CMEM supplemented with insulin (1 μM), dexamethasone (1 μM), $NaHCO_3$ (2.1 g/l), and L-glutamine (4 mM), as reported in Waxman et al. (1990). In these experiments, Primaria® (Falcon, UK) and Nunc tissue culture plates were used in addition to Permanox plates.

In induction experiments, 7 ml of medium containing sodium phenobarbital (0.75

mM) or picrotoxin (0.5 mM) were added to the cells either immediately after attachment or after 48 hr of culture.

2.3 FAZA 967 culture

The FAZA 967 cell line was a kind gift from Prof. Sue Povey, MRC Human Biochemical Genetics Unit and the Galton Laboratory, University College London, London, U.K. Prior to culturing FAZA 967 cells under conditions developed for the primary hepatocytes, these cells were propagated on 100 mm Falcon tissue culture plates, in the presence of EMEM with Hanks' salts, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco BRL, U.K.), 1 % MEM non essential amino acids (Gibco BRL, U.K.), 10 mM HEPES, 10 % foetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were incubated at 37° C, in a humidified atmosphere of 100 % air.

2.4 RNase protection assays

2.4.1 Total RNA isolation

Prior to harvesting, cells were washed three times with ice-cold phosphate buffered saline (PBS; Gibco BRL, UK). Cells were eventually scraped from the plates in 1.5 ml of PBS and recovered by centrifugation for 5 min. in a refrigerated bench centrifuge at 2,000 revolutions per minute (rpm, Heraus bench centrifuge). Pellets were immediately stored at -80° C until ready for analysis. Total RNA was isolated from frozen cell pellets using the RNAzol B kit (AMS Biotechnology Ltd., UK) according to manufacturers instructions. RNA concentration was estimated spectrophotometrically at 260 nm. RNA integrity was checked by means of electrophoresis though an agarose/formaldehyde gel. Gels were prepared by autoclaving (120° C, 20 min) a suspension of 1 g of agarose in 100 ml of MOPS/EDTA (0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). After autoclaving, when the solution had cooled down to ca. 60° C, 5.1 ml of 40 %

solution of formaldehyde (Sigma Chemical Co., U.K.) were added and mixed by gentle stirring. The gel was then poured into a tray that had been previously cleaned with 70 % ethanol and allowed to solidify. Prior to loading, 25 µl of RNA loading buffer and 2 µl of ethidium bromide (10 mg/ml) were added to 3 µl of sample RNA (2.5 µg) [RNA loading buffer: (for 3 ml) 2.25 ml de-ionised formamide, 0.45 ml of 10 x MOPS/EDTA (pH 7.0), 0.72 ml of 40 % formaldehyde, 0.3 ml RNase-free water, 0.3 ml glycerol, 0.24 ml of 10 % (w/v) bromophenol blue]. Samples were then heated at 65° C for 15 min and then cooled on ice for 5 min. Samples were then loaded and, in a horizontal electrophoresis apparatus filled with MOPS/EDTA, electrophoresed at 80 V until the dye had travelled 3/4 of the length of the gel. RNA bands were then visualised on a UV transilluminator and photographed using a Polaroid MP4 camera.

2.4.2 Probe preparation

For CYP2B1 and CYP2B2 specific probes, suitable linearized templates were kindly prepared by Andrew Elia in our laboratory. For the CYP2B common probe, we used the plasmid pBS2B in which a 235 bp *Bam*HI/*Bal*I of a cDNA clone encoding a rat CYP2B2 (Phillips et al., 1983) was inserted between the *Bam*HI/*Eco*RI sites of a Bluescript KS plasmid (Stratagene), (Akrawi et al., 1993). To generate a suitable template for *in vitro* transcription, this plasmid was linearized by digestion with *Bam*HI, then treated for 30 min. at 37° C with proteinase K (50 µg/ml) (Boehringer Mannheim, Germany) in the presence of 1% SDS, extracted once with an equal volume of phenol/chloroform (1:1, v/v), and ethanol precipitated. A radiolabelled "antisense" transcript of the linearized DNA template was generated by using an *in vitro* transcription kit (Stratagene), T3 RNA polymerase (Stratagene) and [α-^{32}P]UTP (800 Ci/mmol, NEN Research Products, U.K.). DNA templates were then removed by digestion with RNase-free DNase I (Stratagene). After ethanol precipitation, the transcripts were purified by electrophoresis through an 8 M urea/6 % polyacrylamide gel.

After exposing the gel to an X-ray film (Fuji-RX) the portion of gel containing the full-length transcript was cut out and placed in 1 ml of a solution containing 0.5 M ammonium acetate, 10 mM EDTA, and 1 % SDS. RNA was eluted by shaking for 2.5 hr at 37° C in an orbital incubator and ethanol precipitated in the presence of 30 μg of carrier tRNA (calf liver tRNA, Boehringer Mannheim, Germany). RNA probes were resuspended to a final concentration of 10^4 cpm/μl in RNase protection hybridization buffer [80 % deionized formamide, 40 mM PIPES (1,4-piperazinediethanesulphonic acid; pH 6.4), 0.4 M NaCl, 1 mM EDTA (Myers et al., 1985), and stored in aliquots at -20°C.

2.4.3 Assays

Hybridizations were performed essentially as described by Myers et al. (1985) in a total volume of 30 μl of RNase protection hybridization buffer that contained 10-20 μg of sample total RNA and 1-5 x 10^4 cpm of radiolabelled antisense transcript. The total amount of RNA was adjusted to 30 μg by the addition of tRNA. RNA present in the hyridization reactions was denatured by heating at 80° C for 10 min, and then allowed to hybridize overnight at 45° C. Single-stranded, unhybridized RNA molecules were digested with RNase A [350 μl/reaction of a solution containing 4 μg/ml RNase A (Boehringer Mannheim, Germany), 10 mM Tris.Cl pH 7.5, 1 mM EDTA, 0.2 M NaCl, 0.1 M LiCl], at 30° C for 1 hr. Following phenol/chloroform (1.1, v/v) extraction and ethanol precipitation, hybrid RNA molecules were denatured in 97 % deionized formamide/2 mM EDTA at 80° C for 5 min, and electrophoresed through an 8 M urea/6 % polyacrylamide gel. Electrophoresis was performed at 18 mA/gel in 1 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). Gels were prepared using Sequagel™ solutions (National Diagnostics Ltd., U.K.) according to manufacturers instructions. To detect the protected RNA species the gel was dried *in vacuo*, and autoradiographed at -80° C with an intensifying screen.

2.4.4 Quantitation of protected mRNA molecules

The protected RNA probe was quantified by densitometric scanning using a BioRad GS-670 imaging densitometer driven by BioRad "Molecular Analyst" software (BioRad, U.K.). Using the method of Little & Jackson (1987) the quantitation of the corresponding mRNA in terms of molecules/cell was determined from a standard curve of undigested probe. The following is an example of the calculations performed. The number of cpm per pmol of UTP (i.e. the molar specific activity of UTP) in the transcription reaction is calculated by dividing the number of cpm/μl (estimated by scintillation counting) by the number of pmol of UTP present in 1 μl of the transcription reaction. Knowing the number of UTPs present in the probe (e.g. 77 UTPs are present in the CYP2B1/2 common probe) we then calculated the number of cpm per pmol of probe (i.e. the molar specific activity of the probe). In the case of the CYP2B1/2 common probe, this was done by dividing the molar specific activity of UTP by 77. Following densitometric scanning of an autoradiograph exposed in the linear response range, the number of pmol of protected probe can be calculated by comparing the band intensity of the protected fragment to the intensity generated by known amounts of undigested probe (standard curve). However, the value obtained must be adjusted in order to take into account the fact that the proteceted probe is shorter than the undigested probe, and it will therefore contain less UTP. In the case of the CYP2B1/2 common probe, the protected probe contains only 67 UTPs, compared with 77 present in the undigested probe. Hence, for the CYP2B probe, it is necessary to multiply the number obtained from the standard curve by 77/67. As the probe is in vast excess over any individual mRNA species, it can be assumed that all the particular mRNA will have hybridized. Hence the number of pmol of the assayed mRNA in the hybridization reaction is now know. By converting this number to moles and multiplying it by Avogadro's number, the number of mRNA molecules can be calculated. From an estimation made at the time we isolated total RNA we know that 5×10^6 cells yielded 20 μg of total RNA. Hence, for a

hybridization reaction containing 20 μg of total RNA, we can express results as mRNA molecules per cell by dividing the total number of mRNA molecules by 5 x 10^6.

2.5 Immunoblotting

2.5.1 Total cell homogenate preparation

Frozen cell pellets were prepared as described above for total RNA isolation. Total cell homogenate were prepared by homogenizing cell pellets in a solution containing 10 mM potassium phosphate (pH 7.25), 1 mM EDTA, 20 % (v/v) glycerol and 3 mM phenylmethanesulphonylfluoride (PMSF). Protein concentration was measured using the Bradford protein assay reagent (Bio-Rad) according to manufacturers instructions, using bovine serum albumin (Bio-Rad) as standard.

Liver cytosolic extracts were kindly prepared by Miss Gilda Debast in our laboratory.

2.5.2 SDS/PAGE

Proteins were separated by electrophoresis through acrylamide gels essentially as described by Laemmli (1970) and as modified by Phillips et al. (1981, 1983).

2.5.2.1 Gel preparation

Separating gels were prepared by mixing Proteogel™ (National Diagnostics Ltd., U.K.) with pH 8.8 buffer [1.5 M Tris-HCl (pH 8.8), 0.4 % (w/v) sodium dodecyl sulphate (SDS)] water, TEMED (N, N, N', N'-tetramethylethylenediamine; Bio-Rad) and ammonium persulphate so that the final gel composition was 10 % acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1 % SDS, 0.075 % (v/v) TEMED and 0.075 % (w/v) ammonium persulphate. Gels with a different acrylamide concentration were prepared by altering the volumes of Proteogel and water. Immediately after pouring, the gels were overlayed with

0.1 % SDS and allowed to polymerize. Once polymerization was completed, the overlay solution from the separating gel was removed and combs inserted. A stacking gel was then poured on top of the separating gel. These gels were prepared by mixing Proteogel with pH 6.8 buffer [0.5 M Tris-HCl (pH 6.8), 0.4 % (w/v) SDS], water, TEMED and ammonium persulphate so that the final gel composition was 3 % acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1 % SDS, 0.1 % (v/v) TEMED and 0.075 % (w/v) ammonium persulphate.

2.5.2.2 Electrophoresis

Total cell homogenates were diluted to a protein concentration of 1 mg/ml in a solution containing 1 % (w/v) SDS, 10 mM EDTA, 10 mM sodium phosphate buffer (pH 7.0), 1 % 2-mercaptoethanol, 15 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue, 3 mM PMSF. Prior to loading, the samples were boiled for 3 min and centrifuged at 10,000 x g for 30 s. In an electrophoresis buffer containing 0.025 M Tris base (Bio-Rad, electrophoresis grade), 0.192 M glycine and 0.1 % SDS, samples were electrophoresed at 100 V until they entered the separating gel. The voltage was then increased to 200 V and electrophoresis was continued until the dye almost reached the bottom of the gel.

2.5.3 Electroblotting and immunoreaction

After electrophoresis, proteins were electroblotted onto a nitrocellulose filter (Hybond C-extra, Amersham International, U.K.). Electroblotting was accomplished overnight in a transfer buffer containing 192 mM glycine, 25 mM Tris base, and 20 % (v/v) methanol, using a Bio-Rad electroblotting apparatus according to manufacturers instructions.

Proteins were detected using rabbit antisera as described in chapter 3, and goat anti-rabbit immunoglobulin G horseradish peroxidase kit (Bio-Rad,U.K.) according to

the manufacturers instruction. In attempting to detect CYP2B1/2 proteins in FAZA 967 cells we also employed a goat anti-rabbit immunoglobulin G alkaline phosphatase kit (Bio-Rad, U.K.).

2.6 Transfection of plasmid DNA by lipofection into primary hepatocytes and FAZA 967 cells

2.6.1 Preparation of plasmid DNA

Plasmid DNA for transfection was prepared using Qiagen 500™ (Qiagen, U.K) columns, as recommended by the manufacturers, and was subsequently de-salted by spin-dialysis, essentially as described in Sambrook et al. (1989). Briefly, the DNA was loaded onto the membrane of a Centricon™ 30 (Amicon, U.K.) spin-column, according to manufacturers instructions, and centrifuged in a Sorvall at 5,000 rpm for 20 min. Sterile, double-distilled water (750 µl) was added to the DNA and centrifuged again at 5,000 rpm for 20 min. This step was repeated three more times. De-salted DNA was finally recovered into an appropriate collection tube by inverting the column and centrifuging at 3,000 rpm for 3 min. DNA concentration was estimated by spectrophotometry at 260nm.

2.6.2 Transfection protocol

After attachment onto 60 mm Vitrogen-coated Permanox plates, cells were washed twice with serum-free William's E medium. After the final wash, 1 ml of serum-free William's E medium, supplemented as described in § 2.2.2, was added to the cells. For each plate, 60 µl of Lipofectin® reagent (Gibco BRL, U.K.) was added to 1.5 ml of serum-free William's E medium with supplements, in an Eppendorf tube. The solution was then briefly vortexed. DNA (10 µg in water; in co-transfection experiments added 8 µg of test plasmid and 2 µg of pCMV-βgal) was then added to the medium containing Lipofectin and mixed by vortexing. The mixture was allowed to stand for 15 min at room

temperature and was then added drop-wise to the cells. Plates were then placed at 37° C in a humidified atmosphere of 95 % air and 5 % CO_2 and incubated for 18 hr. The medium was then removed and the cells washed three times with 1 x PBS. Fresh serum-free William's E medium (7 ml) was then added and the cells kept in culture for 48 hr prior to *in situ* staining with X-gal (5-bromo-4-chloro-3-indolyl-β-galactosidase) or harvesting to assay for luciferase activity. In induction experiments William's E medium was supplemented also with 0.75 mM PB.

2.7 Reporter genes assays

2.7.1 *In situ* staining of cells for β-galactosidase activity

Cells were assayed for β-galactosidase activity by *in situ* staining with X-gal essentially as described in Promega Corporation Technical Bulletin no. 097 as revised in March 1993 (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399 U.S.A). Cells were washed two times with 1 x PBS and fixed by adding 2 ml of a solution containing 0.1 M sodium phosphate buffer (pH 7.0), 1 mM Mg Cl_2 and 0.25 % glutaraldehyde (Sigma Chemical Co., tissue culture grade). After 15 min at room temperature, the glutaraldehyde solution was removed and the cells were rinsed three times with 1 x PBS. A solution containing 0.2 % X-gal (Gibco BRL), 1 mM Mg Cl_2, 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 3.3 mM $K_3Fe(CN)_6$, 60 mM Na_2PO_4, 40 mM NaH_2PO_4 was then added and incubated the cells at 37° C for 60 min. The X-gal solution was revoved and the cells covered with 1 x PBS. Cells were viewed and photographed using a phase contrast microscope.

2.7.2 Luciferase activity assay

Total cell extracts were prepared and luciferase activity assayed using the "Luciferase Assay System" (Promega Corp., U.S.A.), according to the manufacturers instructions. Light emission was detected using an LKB-520 luminometer (Pharmacia,

Sweden). The linear range of the luminometer was determined according to Promega's instructions, using the luciferase assay reagent and purified luciferase (Boehringer Mannheim, Germany).

Chapter three

Results and discussion

When hepatocytes are placed in culture, they rapidly lose the ability to express and induce many drug metabolising enzymes. To overcome these difficulties we decided to take three different approaches to develop a culture system(s) that more closely reflects the situation *in vivo*. These were: 1) to improve the conditions for culturing primary hepatocytes; 2) to analyse hepatoma cell lines and improve conditions for their culture, and 3) to immortalize primary hepatocytes. I shall describe the results obtained with each cell system in sequence.

3.1 Primary hepatocytes

3.1.1 Isolation

A basic protocol for the isolation in high yields of highly viable rat hepatocytes has been available since 1969 (Berry & Friend, 1969) and was subsequently improved in 1973 (Seglen, 1973; Seglen, 1973b). This method is generally referred to as the *two-step collagenase perfusion*. During this procedure, the first step is to destabilize intercellular contacts by removal of calcium. This is accomplished by perfusing the liver with a Ca^{2+}- and Mg^{2+}-free buffered salt solution. Chelators such as EGTA and EDTA can be employed at this stage. The second step involves perfusing the liver with a Ca^{2+}- and collagenase-containing buffer in order to digest the connective tissues of the liver and thus liberate intact cells. The overall procedure is performed at physiological conditions.

However, many factors can influence the success of a hepatocyte isolation procedure and a large number of small variations to the basic protocol have been employed (reviewed in Seglen, 1976).

In our efforts to devise a rat hepatocyte isolation protocol that consistently achieved highly viable cells, we tested several different permutations of the protocol described in (Seglen, 1976). The criterion used to compare results was the percentage of isolated cells that

excluded the dye Trypan Blue (Jauregui et al., 1981). This test is widely used but only measures gross structural integrity, and not *viability* in the strictest sense. Only cells with an intact plasma membrane can prevent the dye from diffusing into the inside of the cell. However, a cell may have internal metabolic lesions or small surface alterations. These will not be revealed by the trypan blue test. For this reason, we used a strict criterion when scoring cells as viable: cells that showed even the slightest nuclear staining were considered "non-viable". In addition, cells that excluded the dye but did not present a refractile appearance and a well-defined outline were also scored "non-viable" (Seglen, 1976).

Technically, the most difficult part of the collagenase protocol is successful perfusion of the liver. We found this factor alone to bear the greatest influence on cell viability. We were able to isolate hepatocytes with a viability >75% by directly cannulating isolated liver lobes (a modification of the procedure described in Strom et al., 1982). However, we could not do so with a high degree of consistency. Also, the yield of dye-excluding cells achieved with this technique was considerably lower than that achieved by perfusion of the whole liver through cannulation of the portal vein. A factor that may have contributed to the variability observed with this method could be the fact that the perfusate was directly pumped into the liver by means of a peristaltic pump. Indeed, differences in flow rate affected perfusion success and best results were achieved using a flow rate of 20 ml/min. Direct pumping of perfusate into the liver may cause fluctuations in pressure inside the liver that could lead to cell damage. By using a three-way bubble trap, according to the specifications employed in Prof. Vera Rogiers laboratory at the Vrije Universiteit of Brussels, Belgium, it was possible to create a system where the perfusate flows into the liver by gravity, rather than by direct pumping (Figure 3.1). This system produces a uniform perfusate flow that is only dependent on the internal capacity of the liver, once the internal diameter of the tubing employed and the height of the bubble trap in relation to the liver are

kept constant. As a consequence, perfusion pressure is kept consistent from one perfusion to the other. The optimum internal diameter of the tubing and the height of the bubble trap from the liver were found in Prof. Rogiers laboratory to be 4 mm and 20 cm, respectively. In this system the peristaltic pump is used only to ensure that the perfusate passes continuously through a heat exchanger, so that it is kept at 37°C. Moreover, by setting the internal flow rate of the system at >50 ml/min it is possible to achieve adequate oxygenation of the buffers even using a very low gassing rate. This is beneficial since we found that a high rate of continuous gassing of the buffers with 95% air and 5% CO_2 contributed negatively to the success of perfusion. Possibly, this is due to the fact that continuous gassing promotes the formation of microbubbles that can clog capillary veins and thus limit the extent of perfusion (Seglen, 1976). Continuous gassing may also lead to the formation of free radicals that can cause damage to cells. Gassing was only used in brief pulses and at a very low rate during perfusion in order to preserve the pH at 7.5, as indicated by a pH indicator (Phenol red) present in the buffers.

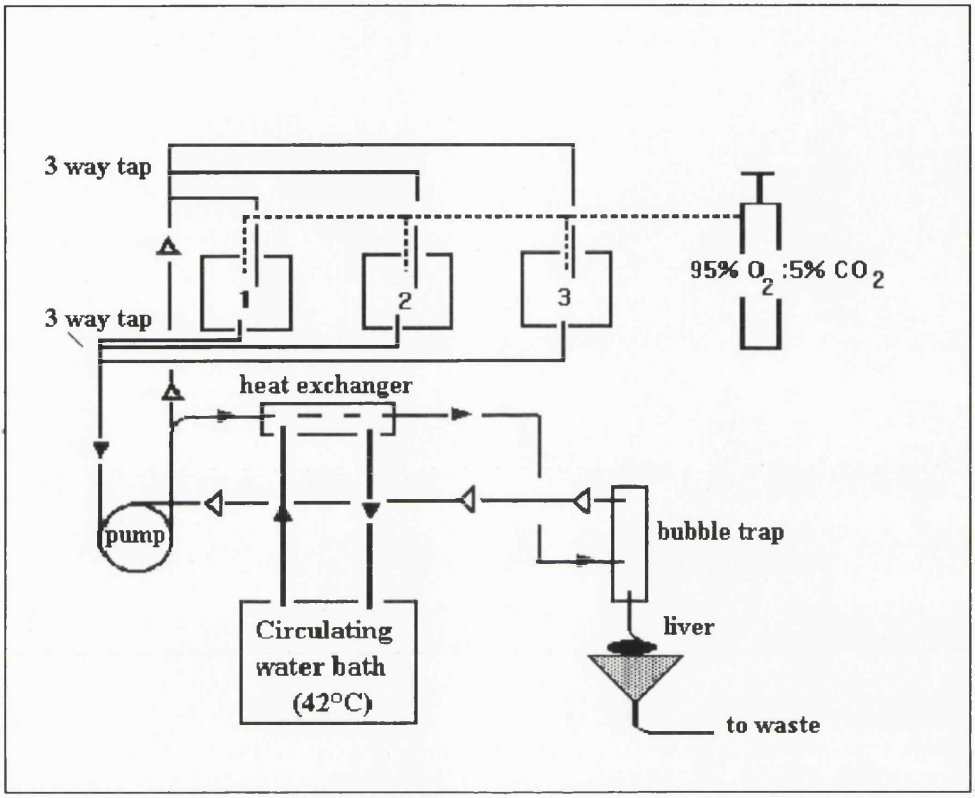


Fig. 3.1 Schematic representation of the perfusion apparatus. (1) reservoir containing perfusate + EGTA; (2) reservoir containing perfusate; (3) reservoir containing perfusate + calcium chloride + collagenase. Filled triangles (▲) indicate the direction of flow of the perfusate going to the bubble trap; hollow triangles (△) indicate the flow of the perfusate from the bubble trap back to the reservoirs."pump" is a peristaltic pump.

Several other factors were found to be critical for a successful liver perfusion. In agreement with Seglen (1976), best results were obtained with a $CaCl_2$ concentration of 5mM and pH at 7.5. Optimal cell dispersion was achieved when buffers were kept at 37°C. However it was still possible to achieve good dispersion at 35°C. This was particularly useful when isolating cells from the livers of transgenic mice (discussed in section 3.4). Addition of Bovine Serum Albumin (BSA) to the dispersal medium, at a final concentration of 2.5%, greatly contributed to the preservation of the viability of hepatocytes during washing steps. No significant difference could be observed between a highly purified form of BSA (fatty acid free, purchased from Sigma Chemical Co.) or a less pure form (fraction V, Sigma Chemical Co.).

Although collagenase is the dispersal agent of choice in most hepatocyte isolation procedures, it has some drawbacks. The collagenase preparations commonly employed for tissue dispersion are rather crude extracts of *Chlostridium hystolyticum*, which contain many proteolytic activities as contaminants. In addition, they are expensive and show considerable batch variation (Howard et al., 1973; Garrison & Haynes, 1973). The proteolytic activities present as contaminants do not aid tissue dispersion (Seglen, 1973 b). Indeed, they may damage proteins on the surface of hepatocytes that could be important for cell attachment and survival. The major contaminating protease activity found in collagenase preparations is trypsin (Seglen, 1973). For this reason trypsin inhibitor was added (0.001 %), with beneficial effect, to the collagenase buffer during the isolation procedure.

To overcome the drawbacks of crude collagenase preparations, perfusion protocols have been deviced that utlize EDTA as the sole dispersal agent. EDTA has the advantage of being relatively cheap, free of proteolytic contaminants, and presents no problems of batch variability. We tried the protocols devised by Meredith (1988), and Bayad et al. (1991), with the only modification being that we omitted the percoll centrifugation step. In both cases,

viability and yield were considerably lower than those achieved with collagenase perfusion. Despite trying several different EDTA concentrations (0.1 to 0.5 mM) and different buffer compositions, we could not improve viability yields significantly above 40%.

The protocol described in chapter two consistently achieves high yields of viable hepatocytes. Typically, a liver isolated from a 200-250 g rat yields >3 x 10^8 dye-excluding cells, with an overall viability >85%.

3.1.2 Culture

On the basis of evidence available at the time this project started (Sinclair et al., 1990; Waxman et al., 1990), we tested several combinations of culture plates and media for their ability to support good cell attachment, morphology and survival of isolated hepatocytes. Comparison of the different culture conditions were done using hepatocytes isolated from the same liver. Best results were obtained when cells were plated on Vitrogen™-coated Permanox® plates (Waxman et al., 1990), in the presence of Williams' E medium (Sinclair et al., 1990) (Fig. 3.2). Insulin (1.7 μM) and dexamethasone (0.1 μM) were the only hormones added to the medium. The presence of dialyzed foetal calf serum (FCS) during attachment was found to be beneficial. Cells kept under these conditions could survive in culture for at least seven days, with no major signs of deterioration. The ability of these culture conditions to support longer cell survival, however, was not monitored. Good cell attachment and survival were not as reproducible on uncoated Permanox® plates (Fig. 3.3) as they were on Vitrogen-coated Permanox plates, suggesting that the matrix makes a strong contribution to the overall stability of hepatocytes in culture. High cell density was also found to contribute strongly to the stability of these cells in culture. This is in agreement with previously published observations (Nakamura et al., 1983). Best results were obtained when cells were seeded at a density of 5 x 10^6 cells/60 mm plate, in a volume of 2 ml.

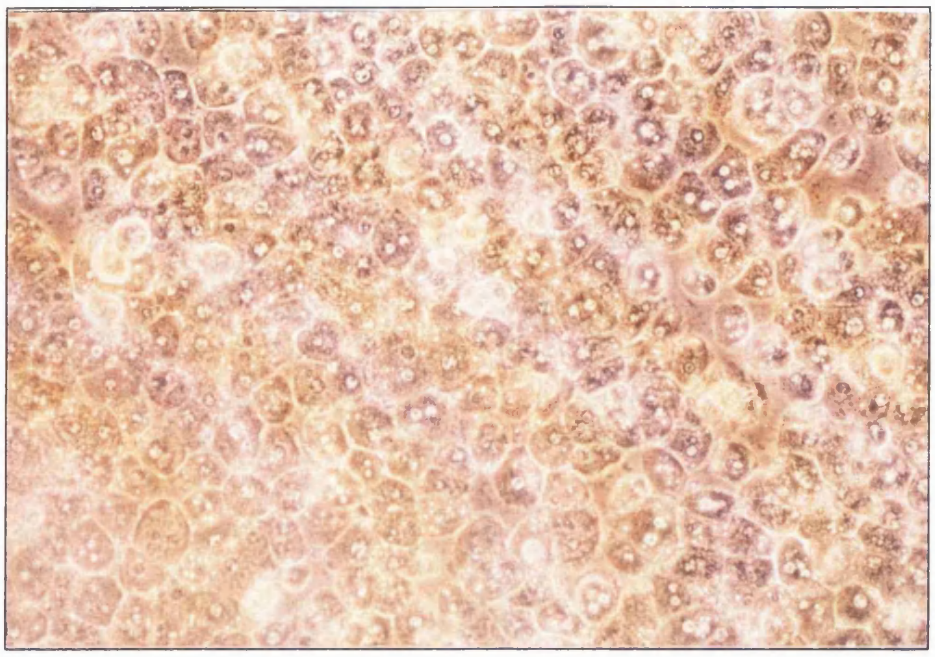


Fig. 3.2 Light photomicrograph of adult rat hepatocytes cultured on Vitrogen™-coated Permanox® plates, after 4 days in culture (magnification x 40).

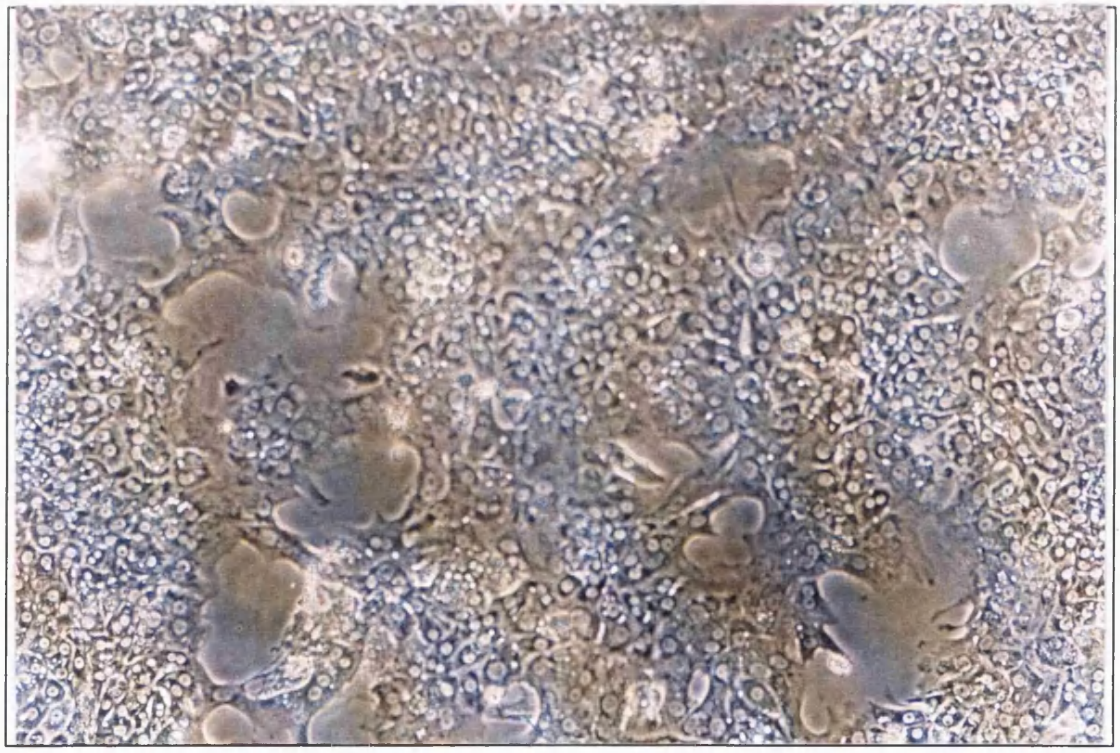


Fig. 3.3 Light photomicrograph of hepatocytes plated on uncoated Permanox® plates, after four days in culture (magnification x 25).

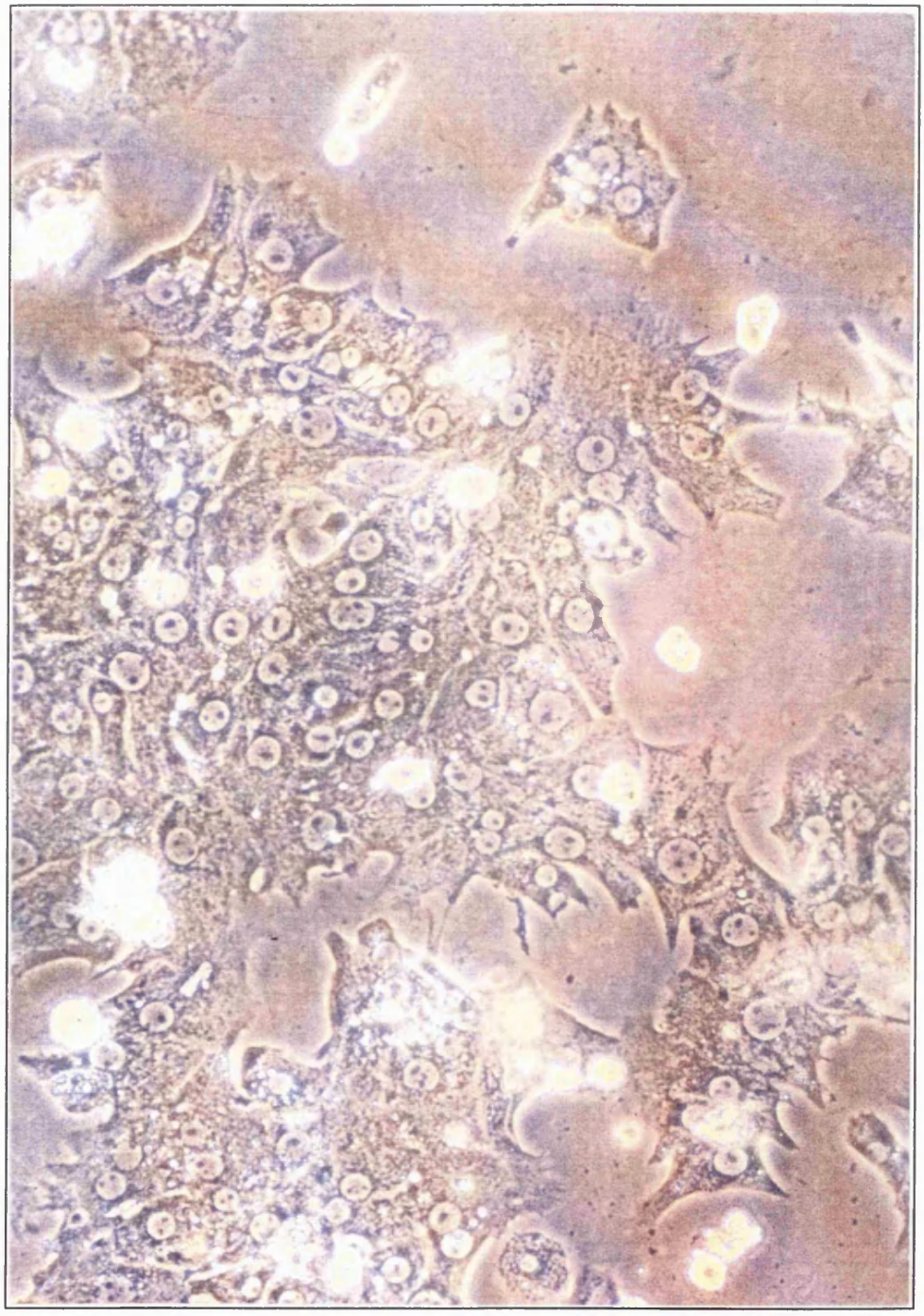


Fig. 3.4 Light photomicrograph of adult rat hepatocytes plated on uncoated Primaria® dishes, after four days in culture (original magnification x 40).

We could achieve good attachment and cell survival neither when cells were plated on uncoated Falcon Primaria® dishes (Sinclair, 1990) (Fig. 3.4), nor when hepatocytes were plated in Chee's Modified Eagles' Medium (CMEM) (Waxman, 1990).

3.1.3 Expression of CYP2B1/2 mRNAs

Having established culture conditions that promoted good cell attachment, morphology and survival (section 2.2.2), we went on to test whether these conditions supported the expression of drug-metabolizing enzymes. Initially, we concentrated in particular on the expression and induction of *CYP2B* genes. As already mentioned in section 1.1, the expression and induction of these genes constitute a good marker for hepatocyte differentiation and are normally lost in cultured hepatocytes. Therefore, it is likely that a cell system capable of supporting the expression and induction of these genes would be capable of supporting also the expression of other drug-metabolising enzymes.

The expression and induction of members of the *CYP2B* gene subfamily were investigated through the use of a quantitative RNase protection assay performed on total cellular RNA. The sequence of the antisense RNA probe used in these experiments is identical with those of all the CYP2B1 and 2B2 variants, but it is distinct from that of CYP2B3 (Labbé et al., 1988). Thus, experiments performed using this probe would quantify all know CYP2B1 and 2B2 mRNAs, but not 2B3 (Akrawi et al., 1993). Prior to hybridization, total RNA was checked for integrity by means of electrophoresis through a formaldehyde/agarose gel, containing ethidium bromide. Following electrophoresis, RNA could be easily visualized by exposing the gel to ultra violet (UV) light. An example of such a gel is shown in Fig.3.5.

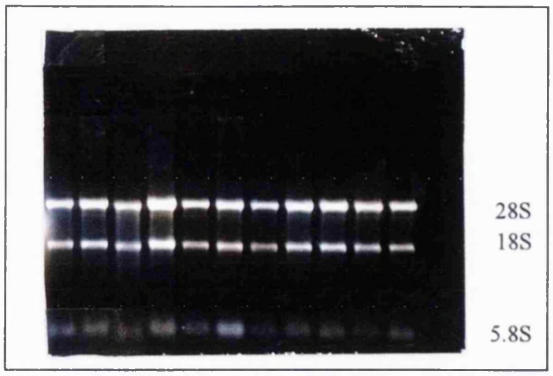


Fig. 3.5 Example of a formaldehyde/agarose gel. Each lane contains 2.5 µg of total cellular RNA isolated from cultured hepatocytes. Three bands corresponding to 28S, 18S and 5.8S rRNA molecules are clearly visible, indicating that the total RNA is intact.

3.1.3.1 Induction by phenobarbital of CYP2B mRNAs

In initial non quantitative experiments, we investigated the expression and induction by phenobarbital (PB) of CYP2B1/2 genes in hepatocytes cultured for a total period of 48 and 96 hours (Fig. 3.6, tracks d-g), and compared it to that observed in rat liver (Fig. 3.6, tracks a and b) and in freshly isolated hepatocytes (Fig. 3.6, track c).

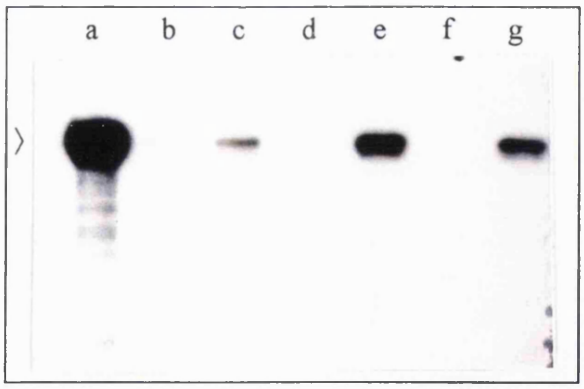


Fig. 3.6 RNase protection assays of CYP2B1/2 mRNAs. Total RNA was extracted from rat liver (a, b), freshly isolated hepatocytes (c), and hepatocytes cultured for 48 (d, e) and 96 (f, g) hours. RNA samples in tracks e and g were isolated from cultures treated with 0.75 mM PB; those in tracks d and f from untreated cultures. RNA in track a was extracted from the liver of a rat treated with PB and in track b from an untreated rat. Each hybridization reaction contained 20 µg of total RNA and 1 x 10^4 cpm of radiolabeled antisense RNA complementary to both CYP2B1 and 2B2 mRNAs. The arrowhead indicates the fully protected 235 nt fragment.

In untreated cultures, the expression of CYP2B1/2 mRNAs remained constant between 48 and 96 hours (d, f), albeit at a level lower than that observed in freshly isolated hepatocytes (c) and in untreated rat liver (b). When hepatocytes were incubated in the presence of phenobarbital at a concentration of 0.75 mM (tracks e and g), the expression of CYP2B1/2 mRNAs was increased approximately 60-fold compared to untreated cultures (tracks d and f). The amounts of CYP2B1/2 mRNAs present, although not identical to those observed in phenobarbital-treated rat liver (track a) were substantial. Because in this experiment PB was added to hepatocyte cultures immediately after attachment it is possible that the observed increase in the steady-state levels of CYP2B1/2 mRNAs was a consequence of the stabilization of mRNA transcripts that were already present in the cells. We performed therefore experiments in which cells were left in culture for 48 hr prior to addition of PB. The barbiturate was then left in the cultures for a further 48 hr. The induction of CYP2B1/2 mRNAs (Fig. 3.7) was approximately 65-fold and, hence, comparable to that observed when cells were treated with PB immediately after attachment.

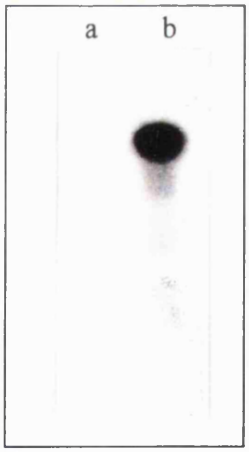


Fig. 3.7 RNase protection assay performed on total RNA (20 μg) isolated from primary hepatocytes. Cells were left either untreated (track a) or were treated with 0.75 mM PB, 48 hr after attachment (track b). Cells were kept in culture for a total of 96 hr.

To show that RNase protection assays were performed under conditions of probe excess, we performed a "two-point" assay, in which hybridizations were done in the presence of either 10 or 20 μg of total RNA isolated from rat hepatocyte cultures. If the assay conditions are such that the probe is present in molar excess over target mRNA molecules, the number of protected molecules should be directly proportional to the amount of total RNA present in the hybridization mixture. An example of such an experiment is shown in Fig. 3.8, panel A. Densitometric analysis of the autoradiograms (Fig. 3.8, panel B) showed that the CYP2B1/2 mRNA molecules protected when the hybridization is performed in the presence of 10μg of total RNA ("C10" = 0.1 molecules/cells and "P10"= 8.5 molecules/cell) were approximately half of those protected when the hybridization is performed in the presence of 20 μg of RNA ("C20" = 0.25 and "P20" = 17.8 molecules of mRNA per cell), confirming that the assay conditions allow protection of all the target mRNA molecules present in the hybridization mixture. The results of several RNase protection assays of the amounts of CYP2B1/2 mRNAs present in untreated and phenobarbital treated cells are shown graphically in Fig. 3.9. The concentration of CYP2B1/2 mRNAs remained constant in untreated cultures for four days (Fig. 3.9, C48 and C96) at a mean value of 0.3 (± 0.1) molecules/cell . When rat hepatocytes were cultured in the presence of phenobarbital (Fig. 3.9, P48 and P96), the concentration of CYP2B1/2 mRNAs increased to a mean value of 19.7 (± 4.9; >66-fold induction) molecules/cell after 48 hr in culture (Fig. 3.9, P48), but dropped to 6.6 (± 0.69) molecules/cell after a further 48 hr (Fig. 3.9, P96). The fold induction of CYP2B1/2 mRNAs in hepatocytes cultured for 48 hr in the presence of phenobarbital is very similar to the fold increase observed *in vivo* (Fig. 3.9, LC and LP). The number of CYP2B1/2 mRNA molecules expressed in treated cultures is approximately 50 % of the number of molecules expressed in PB-treated rat liver (Fig. 3.9, LP).

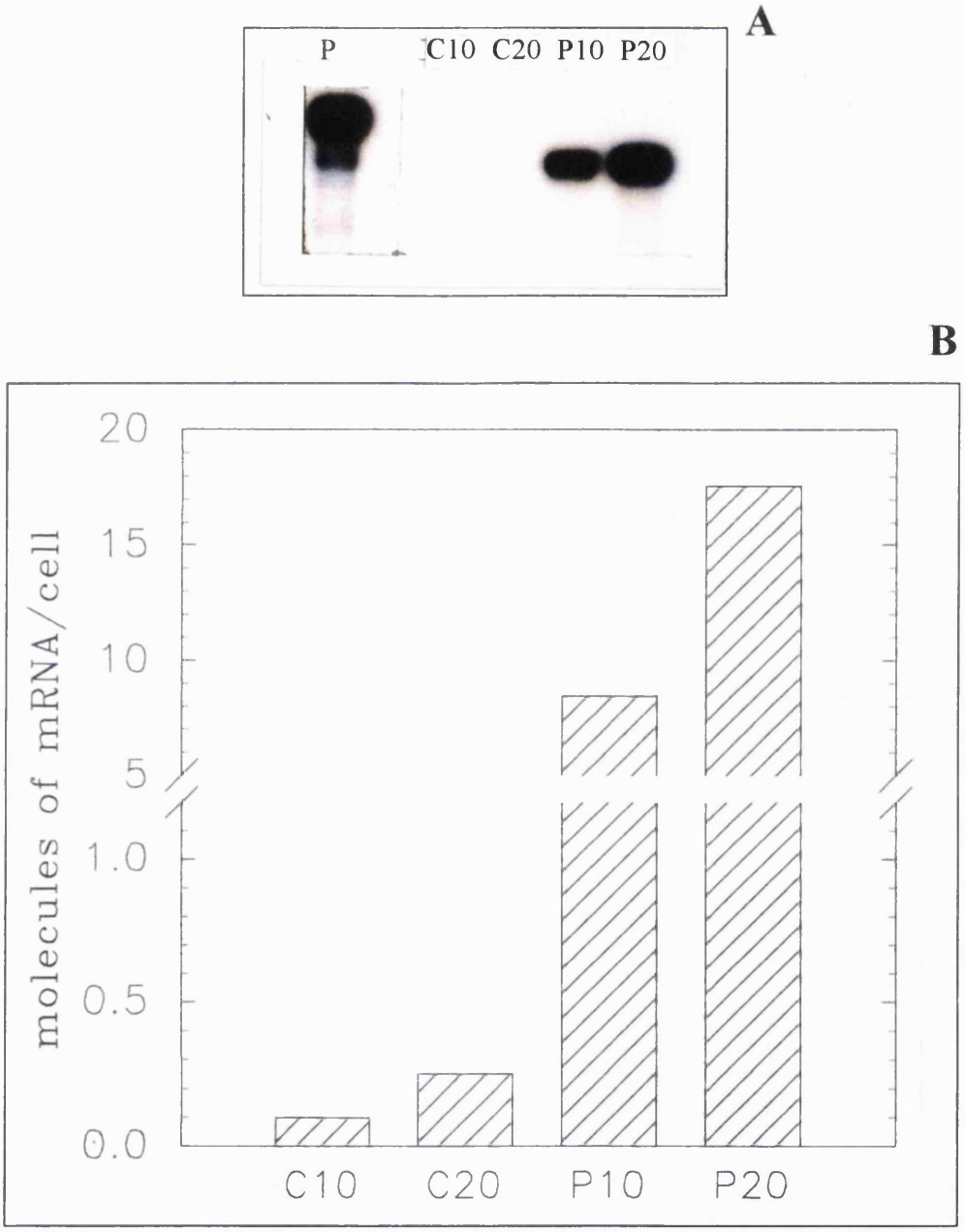


Fig. 3.8 "Two-point" RNase protection assay of CYP2B1/2 mRNAs. Panel **A** shows an example of a two-point assay. Hybridization reactions contained either 10 (C10 and P10) or 20 μg (C20 and P20) of total RNA. RNA samples in tracks P10 and P20 were isolated from cultures treated for two days with 0.75 mM PB, 48 hr after attachment. RNA samples in tracks C10 and C20 were isolated from untreated cultures. Track P shows undigested probe. Panel B is a graphical representation of the results derived from the densitometric analysis of the autoradiogram shown in panel A. Results were calculated in terms of molecules of mRNA per cell as described in section 2.4.4.

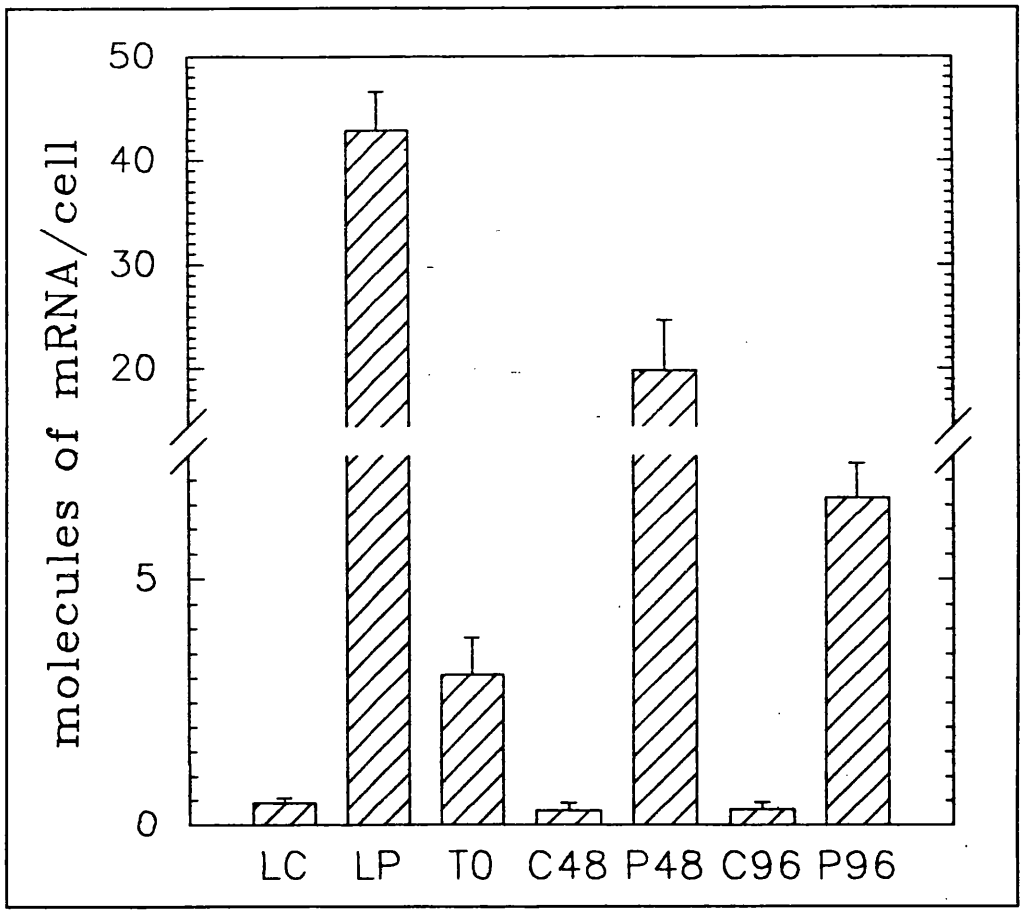


Fig. 3.9 Graphical representation of the results of several RNase protection assays of CYP2B1/2 mRNAs. LC: untreated rat liver; LP: PB-treated rat liver; T0: freshly isolated rat hepatocytes; C48 and C96: untreated hepatocytes cultured for 48 hr and 96 hr respectively; P48 and P96: PB-treated hepatocytes cultured for 48 hr and 96 hr, respectively. Filled bars represent the mean ± SD. For samples LC, LP, T0, C96 and P96, n = 3, for samples C48 and P48, n = 5. $P < 0.0$(C48 versus P48; C96 versus P96)

With the exception of Akrawi et al. (1993), the results of previous investigators were non quantitative (Schuetz et al., 1988; Sinclair et al., 1990; Waxman et al., 1990; Kocarek et al., 1993; Sidhu et al., 1993). A direct comparison of our results with those obtained by these investigators, using different culture conditions, is therefore difficult. However, in terms of fold induction and expression levels, relative to rat liver or freshly isolated hepatocytes, our results compare very favourably. Guzelian P. S. and collaborators (Schuetz

et al., 1988; Kocareck et al., 1993) demonstrated induction by phenobarbital of CYP2B1/2 mRNAs in primary rat hepatocytes cultured on a reconsituted extracellular matrix (Matrigel; Ben-Ze'ev et al., 1988) in Waymouth medium. However, the levels of expression of CYP2B1/2 mRNAs in phenobarbital-treated cultures were only ≈ 10 % of the levels expressed in rat liver (Schuetz et al., 1989) and ≈ 60 % of those observed in freshly isolated hepatocytes (Kocarek et al., 1993). Sinclair et al. (1990) demonstrated that when rat hepatocytes are cultured in William's E medium, CYP2B1 and CYP2B2 proteins were induced by phenobarbital regardless of whether cells were maintained on Vitrogen or Matrigel. This was true also of benzyloxiresorufin dealkylase (BZROD) and pentoxyresorufin dealkylase (PROD), two catalytic activities associated with CYP2B1 and CYP2B2 (Burke et al., 1985). Unfortunately, in this investigation the amounts of anti-CYP2B1/2 immunoreactive proteins in PB-treated rat liver were not measured. It is not possible therefore, to compare the fold induction of CYP2B1/2 proteins in cells cultured under these conditions. In any case, BZROD and PROD activities were only 40 % and 30 %, respectively, of the activities measured in PB-treated rat liver. Expression of CYP2B1/2 mRNAs was also not measured in this report.

Waxman et al. (1990) cultured hepatocytes on Vitrogen-coated plates in serum-free CMEM medium. In PB-treated cells cultured under these conditions, he showed by immunoblotting that the amounts of CYP2B1/2 proteins were comparable to those observed in PB treated rat liver. In addition, he showed that in these cells the CYP2B1/2 related 16β-hydroxylase activity was comparable to that measured in PB-treated rat liver. Unfortunately, because the expression of CYP2B1/2 mRNAs was not measured, it was not possible to determine whether to the increase in the expression CYP2B1/2 proteins observed in these cells, corresponded to an increase in the expression of CYP2B1/2 mRNAs. Indeed, Sidhu et al. (1993) reported that in cells cultured under these conditions it was not possible to

observe a strong phenobarbital-mediated increase in the expression of CYP2B1/2 mRNAs despite observing a strong increase in the expression of CYP2B1/2 proteins and associated catalytic activities. This observation suggested that mRNA and protein stabilisation strongly contributed to the induction of CYP2B1/2 proteins in these cells. Interestingly, in the same report, Sidhu et al. also desribed the effect, on the expression of *CYP2B1/2* genes, of ovelaying with Matrigel, cells plated on Vitrogen-coated plates. When cultured in William's E medium supplemented with PB and dexamethasone, induction of CYP2B1 mRNA in the cells "sandwiched" between the two extracellular matrices was 1.5-fold higher than that observed in PB-treated rat liver. In contrast, the fold induction of CYP2B2 mRNA was only ≈60 % of that observed in PB treated rat liver. Indeed, the fold induction of the CYP2B1 mRNA in this cell system is the highest so far reported. Unfortunately, results were only shown, in a graphical representation, as a "percentage induction relative to PB induced liver", providing no indication about the amounts of CYP2B1 mRNA expressed in untreated cultures. It is possible therefore that, this unusually high fold induction of CYP2B1 mRNA may be due to a very low expression of CYP2B1/2 mRNAs in untreated cells. Another factor may have contributed to the large fold induction observed in this cell system. The expression of CYP2B mRNAs was measured using slot blots and oligonucleotide probes. Because a very high percentage of nucleotides in the CYP2B1 and CYP2B2 mRNAs are identical (in the most divergent part they still share 90.5 % nucleotide sequence identity), it is likely that this technique may not be specific enough to be able to distinguish with 100 % efficiency between the two mRNAs. Hence, CYP2B2 mRNA may have contributed to the signal obtained for the CYPB1 mRNA, and vice versa.

Our results appear to be in broad agreement with results obtained by Akrawi et al. (1993) who reported that, in the more complex system in which hepatocytes are co-cultured with biliary hepithelial cells, hepatocytes express between 10.5 and 50 molecules of

CYP2B1/2 mRNAs per cell when cultured in the presence of phenobarbital, as measured by quantitative RNase protection assays. In untreated cells, the number of CYP2B1/2 molecules was 3 to 3.5. The fold induction of CYP2B1/2 mRNAs in this system was 12-15-fold.

It was interesting to note that freshly isolated hepatocytes (Fig. 3.9, "T0") expressed CYP2B1/2 mRNAs in amounts higher than those observed in untreated rat liver. Several factors may contribute to this effect. It may be a consequence of the fact that freshly isolated cells are enriched for parenchymal cells ("freshly isolated hepatocyte" in this study were those obtained after centrifugation and washing steps), whereas in the liver only ≈ 60 % of cells are parenchymal (Gebhardt, 1992). Additionally, the isolation procedure itself may have caused an induction in the expression of *CYP2B1/2* genes. Hepatocytes, during a collagenase perfusion, are subjected to stress: chemical, mechanical and oxidative. These kinds of stresses have been shown to stimulate the expression and/or activity of several liver-enriched transcription factors (C/EBP α, β, and δ; NF-κB and related transcription factors, glucocorticoid receptor), in a very rapid fashion, with the consequent increase in expression of several liver proteins (Acute Phase Response; reviewed in Baumann, 1989; Ciliberto, 1989; Ciliberto et al., 1993; Fey & Gauldie, 1990). Many of the *cis*-acting elements of these liver-specific transcription factors have been located within the 5'-flanking region of the *CYP2B2* promoter (Shephard et al., 1994). It is possible to concieve therefore that the stress caused by the isolation procedure may stimulate the expression of the CYP2B1/2 genes. Finally, the barbiturate sodium pentobarbital used to anaesthetise the animals during the perfusion may itself induce *CYP2B1/2* genes (Okey, 1990). This latter possibility, however, is more improbable, since the time required for the barbiturate to induce *CYP2B1/2*, at least *in vitro* (Sidhu et al., 1993) is longer than the time required for the isolation procedure to be completed (~ 40 min.). For the reasons mentioned above, the levels of expression of drug-

metabolizing enzymes (of *CYP2B1/2* genes in particular) observed in freshly isolated hepatocytes cannot be considered as true representative of the levels of expression that occur in the liver. We have therefore excluded from subsequent experiments any measurement on the expression of drug-metabolizing in freshly isolated hepatocytes.

Interestingly, in untreated cultures, the number of detected molecules was below 1. This is possibly a consequence of the fact that not all plated cells are expressing CYP2B1/2 mRNAs. This could be due to: 1) hepatocyte preparations, although highly enriched for parenchymal cells, are never 100% homogeneous; as a consequence they are often contaminated by non-parenchymal cells; 2) cells that are located at the borders of the plate, and therefore are not completely surrounded by other cells, may not express CYP2B1/2 mRNAs if, as expected, cell-cell contact is required for maximal expression of adult-liver functions (Nakamura et al., 1983); 3) not all cells on the plate are healthy and differentiated.

Having established that in rat hepatocytes, cultured in the presence of William's E medium on Vitrogen-.coated Permanox plates, CYP2B1/2 mRNAs were substantially inducible by phenobarbital, we wished to determine the concentrations and inducibility of mRNAs encoding individual members of the CYP2B subfamily, namely CYP2B1 and CYP2B2. For this purpose we employed the RNase protection assay with antisense RNA probes designed specifically to distinguish between CYP2B1 and CYP2B2 mRNAs. These probes were prepared by Andrew Elia in our laboratory and had been previously tested for their specificity (data not shown). Unfortunately, we cannot be sure whether these probes can recognize all CYP2B1 and CYP2B2 variants. The results of these experiments therefore cannot be considered quantitative. An example of such an experiment is shown in Fig. 3.10. CYP2B1 mRNA was highly inducible by PB (≈ 66-fold) in hepatocytes cultured on Vitrogen-coated Permanox plates (Fig. 3.10, "CYP2B1" track b). Expression of CYP2B1 could be detected also in PB-treated hepatocytes plated on uncoated Permanox plates (Fig.

3.10, "CYP2B1" track a), however the amounts of CYP2B1 mRNA in these cells were at least 10-fold less than the amounts detected in PB-treated cells cultured on Vitrogen-coated plates. Unfortunately, CYP2B1 mRNA was undetectable in untreated hepatocytes plated on uncoated Permanox plates (data not shown). Hence, it was not possible to estimate the fold-induction of CYP2B1 mRNA in this cell system. To a lesser extent (≈ 5-fold) CYP2B2 mRNA also was inducible in hepatocytes cultured on Vitrogen-coated plates (Fig. 3.10, "CYP2B2" track b), whereas it was undetectable in hepatocytes plated on uncoated plates. In agreement with observations made *in vivo* (discussed in section 1.2.1.1), in untreated cells plated on Vitrogen coated plates, the amounts of CYP2B2 mRNA were approximately 2-fold more than the amounts of CYP2B1 mRNA. In PB induced rat liver (Fig. 3.10 tracks d), the ratio of CYP2B1 to CYP2B2 mRNAs was approximately four-fold smaller than in PB treated hepatocytes (Fig. 3.10 tracks b). This may be due to the fact that the culture conditions developed during the course of this investigation favour the induction of CYP2B1 mRNA expression. However, at least in part, this observation may be an artifact of the assay employed. Unfortunately, the probes used in this assay protect many bands of different mobility. The contribution of these bands to the overall signal for the full-length protected fragment is difficult to estimate. Further investigations, using different probes, are therefore necessary to precisely establish the expression of CYP2B1 and CYP2B2 mRNAs in hepatocytes cultured under conditions developed during the course of this study.

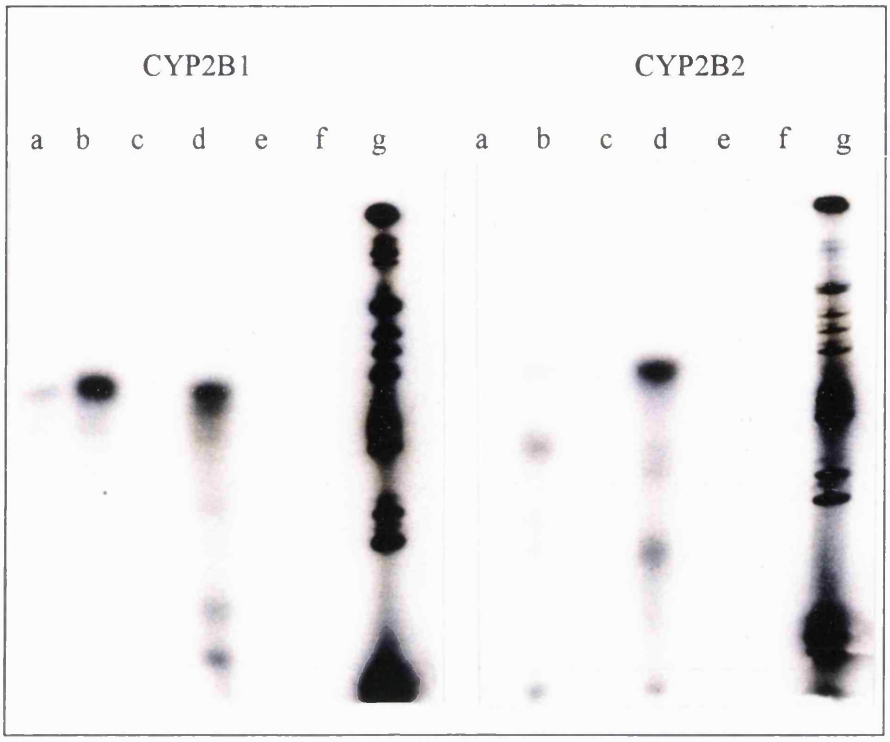


Fig. 3.10 RNase protection assay of CYP2B mRNAs using probes specific either for CYP2B1 or CYP2B2. Tracks a-c contain 20 μg of total RNA isolated from cultured hepatocytes: a) hepatocytes plated on uncoated Permanox plates cultured for 48 hr in the presence of PB; b) hepatocytes plated on Vitrogen-coated Permanox plates cultured for 48 hr in the presence of PB; c) hepatocytes plated on Vitrogen -coated Permanox plates cultured in medium lacking PB. Tracks e and d contain 10 μg of total RNA isolated from untreated and PB-treated rat liver, respectively. Track f contains 30 μg of tRNA and track g shows molecular mass standards (1 kb ladder, Bethesda Research Laboratories).

3.1.3.2 Induction by picrotoxin of CYP2B mRNAs

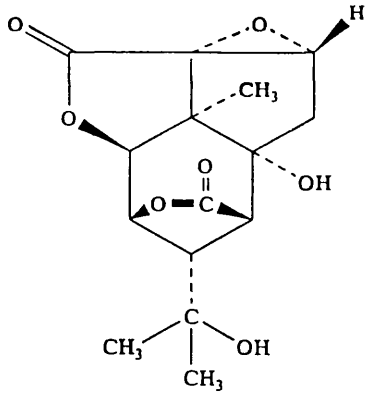
The molecular mechanisms through which phenobarbital stimulates the transcription of CYP2B1/2 genes are as yet unsolved (current knowledge on the subject is reviewed in Waxman, 1992). Because of the fact that phenobarbital is a lipophilic compound capable of entering cells freely, and because induction by this compound shows a saturable dose curve both *in vivo* (Lubet et al., 1985) and *in vitro* (Kocarek et al., 1990), it is possible to speculate that the induction of CYP2B1/2 genes may be mediated by an intracellular receptor. The observation that PB induction exhibits tissue specificity (e.g. Omiecinski et al., 1986; Christou et al., 1987; Traber et al., 1990) is also consistent with this hypothesis. The fact that numerous biochemical investigations (e.g. Tierney & Bresnik, 1981) have failed to detect a protein capable of interacting with PB does not necessarily imply that such a protein does not exist. It is possible in fact that the barbiturate has a very low affinity for its receptor or that this receptor is expressed in very low amounts such that classical biochemical techniques are not sensitive enough. Indeed, a receptor capable of a low affinity interaction with phenobarbital already exists in nature. This protein is the $GABA_A$ receptor. Phenobarbital acts as a sedative and an antiepileptic drug (Brodie, 1990) by interacting in the brain with this receptor at a specific binding site. This interaction causes the opening of an associated chloride channel with the consequent hyperpolarization of nerve cells (reviewed in Olsen & Venter, 1986). It is conceivable to think that if an intracellular PB-receptor does exist, it would share sequence similarity with the GABA receptor, at least in the barbiturate-binding domain. To test this hypothesis, we decided to investigate the effects on CYP2B1/2 expression of Picrotoxin (the structures of the two components of picrotoxin, picrotin and picrotoxinin, are shown in Fig. 3.11). This compound is a plant extract that has been shown to antagonise the effects of barbiturates on the GABA receptor (Hill, 1972) by binding to a site very close (if not identical) to that for barbiturates (Sigel et al., 1990). Our hypothesis

was strengthened by the fact that during the course of this study Yamada et al. (1993) showed that picrotoxin induced the expression of CYP2B1/2 proteins in rats and augmented the activity of several other phenobarbital-inducible liver enzymes, including glutathione S-transferases.

PICROTIN

Non Toxic component of Picrotoxin

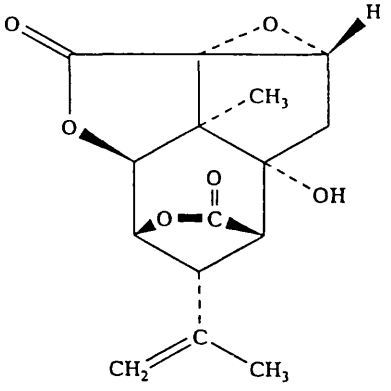
Mol. Wt. 310.29



PICROTOXININ

Toxic Component of Picrotoxin

Mol. Wt. 292.28



PICROTOXIN

ALSO KNOWN AS COCCULIN, IS THE BITTER PRINCIPLE ISOLATED FROM THE SEED OF *ANAMIRTA COCCULUS*. IT IS A MOLECULAR COMPOUND OF ONE MOLE OF PICROTOXININ AND ONE MOLE OF PICROTIN.

THERAPEUTIC USE: CENTRAL AND RESPIRATORY STIMULANT
CNS STIMULANT, ANTIDOTE TO BARBITURATES.

Fig. 3.11 Chemical structure of picrotoxin. This compound has no apparent structural similarity to phenobarbital or other PB-like inducers (Fig. 1.10), other than it is lipophilic.

Using the RNase protection technique, we investigated the effects of picrotoxin on the expression of CYP2B1/2 mRNAs in cultures of primary rat hepatocytes. Cells were cultured for 48 hr either in the absence (Fig. 3.12, panel A, track e) or in the presence of different concentrations of picrotoxin (Fig. 3.12, panel A, tracks a-d). Even at the highest concentration tested (2 mM), picrotoxin did not seem to cause any major damage to cells that could result in an apparent morphological change, at least during a 48 hr incubation period. CYP2B1/2 mRNAs were found to be highly inducible by picrotoxin. Densitometric analysis of the autoradiogram shown in panel A (Fig. 3.12, panel B) showed that highest induction of CYP2B1/2 mRNAs was achieved when cells were cultured in the presence of 0.5 mM picrotoxin (panel A, track d). Interestingly, induction levels were significantly lower at higher concentrations (tracks a-c). A possible explanation may be that although no apparent morphological alterations could be observed in cells cultured in the presence of picrotoxin, it is possible that concentrations higher than 0.5 mM may interfere with cellular functions. Alternatively, induction by this compound of *CYP2B1/2*, like induction by PB, is dose-dependent (Kocarek et al., 1990; Waxman et al., 1990).

Having established that 0.5 mM was the concentration at which picrotoxin best induced the expression of CY2B1/2 mRNAs, we used this concentration in subsequent experiments.

In order to gain clues as to whether picrotoxin and phenobarbital induce the expression of CYP2B1/2 mRNAs via a similar mechanism we investigated the time course of induction by picrotoxin (Fig. 3.13, tracks b, e, j, m) and phenobarbital (Fig. 3.13, tracks c, f, i, l) of these mRNAs. The inducers were added to the cells immediately after attachment and the expression of CYP2B1/2 mRNAs was measured at different times during culture. Induction of CYP2B1/2 mRNAs was already observable after 4 hr of culture in the presence of either picrotoxin (Fig 3.13, track j) or phenobarbital (track i). After 8 hr in culture, both

compounds (PCX: track b; PB: track c) had already increased the expression of CYP2B mRNAs. The data obtained from the densitometric analysis of the experimental results shown in Fig. 3.13 and others were pooled and represented graphically in Fig. 3.14. No significant difference could be observed in the early time course of induction by picrotoxin and by phenobarbital of CYP2B1/2 mRNAs, and the curves of induction by the two compounds, generated by plotting mRNAs concentrations versus time, indicate that no major differences exist between the kinetics of induction by picrotoxin and that by phenobarbital, suggesting that both compounds may stimulate the expression of CYP2B1/2 mRNAs via a similar mechanism.

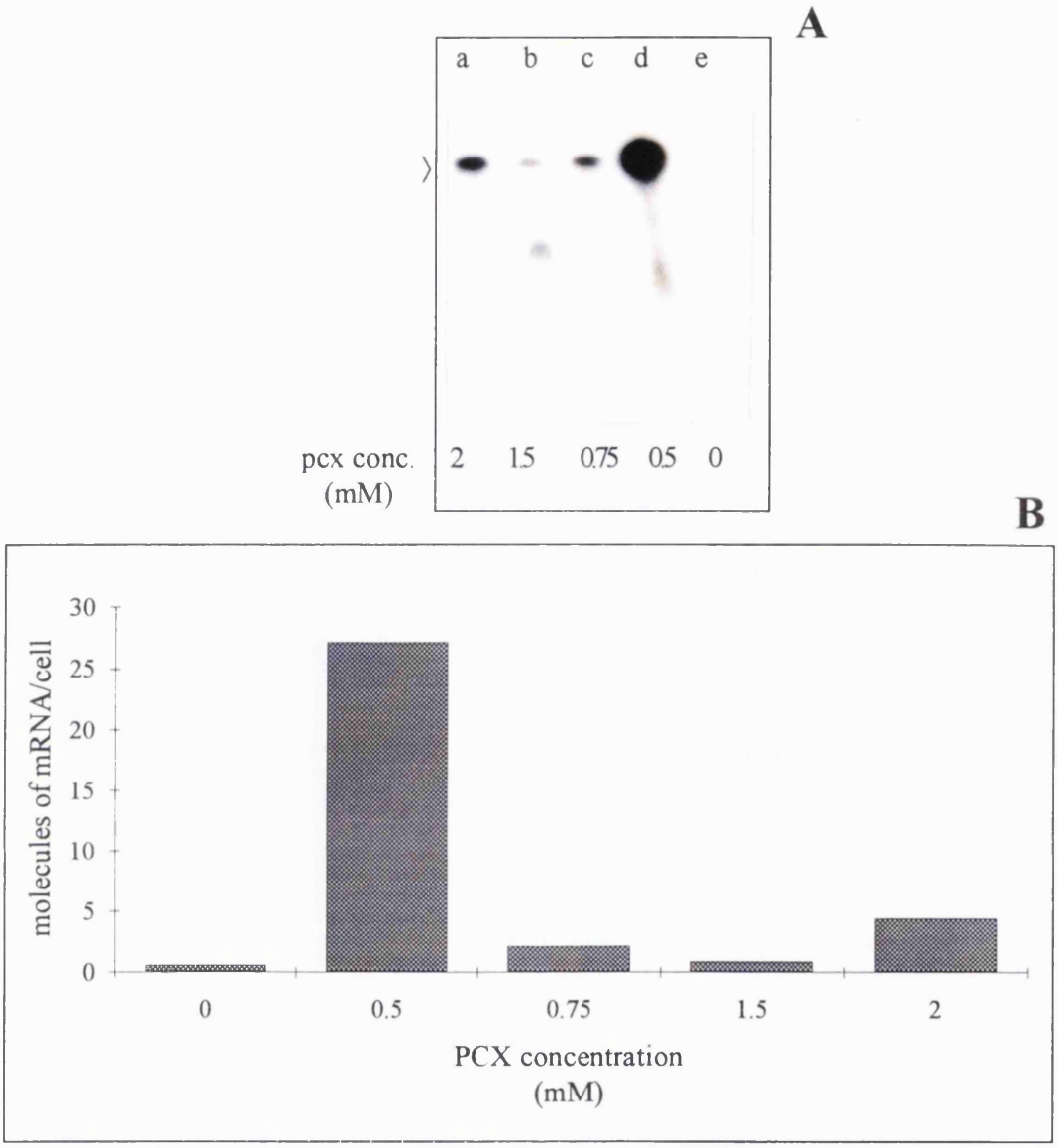


Fig. 3.12 Effect of picrotoxin on the expression of CYP2B1/2 mRNAs in primary hepatocytes. (**A**) RNase protection of CYP2B1 and 2B2 mRNAs in 20 μg of total RNA isolated from hepatocytes cultured for 48 hr in the presence of either 0, 0.5, 0.75, 1.5, or 2.0 mM pcx. The arrowhead indicates the fully-protected fragments. (**B**) Graphical representation of the results shown in panel A.

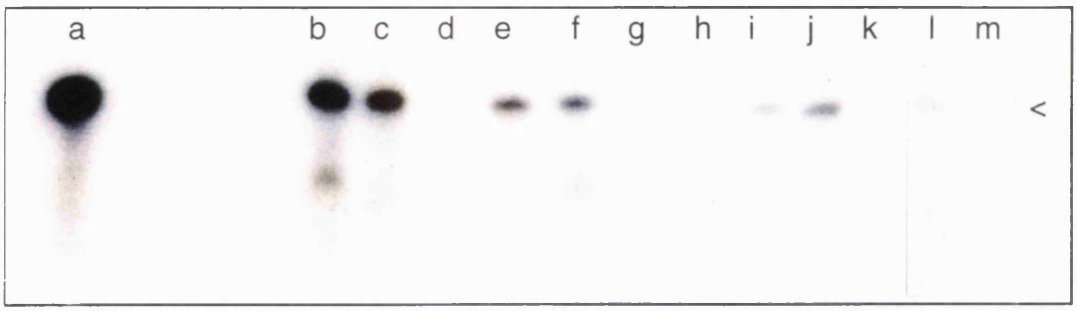


Fig. 3.13 RNase protection of CYP2B1/2 mRNAs: comparison of the effects of phenobarbital and picrotoxin on the time course of expression and induction of CYP2B1/2 mRNAs. After attachment, the medium was changed with fresh, either lacking (d, g, h, k), or supplemented with PB (c, f, i, l), or PCX (b, e, j, m). Total RNA (20 μg) was isolated from hepatocytes cultured for 8 hr (b-d), 6 hr (e-g), 4 hr (h-j), and 2 hr after the change of medium. Track a is a positive control for CYP2B expression, total RNA was isolated from the liver of a rat treated with PB. The arrowhead indicates the fully-protected fragment.

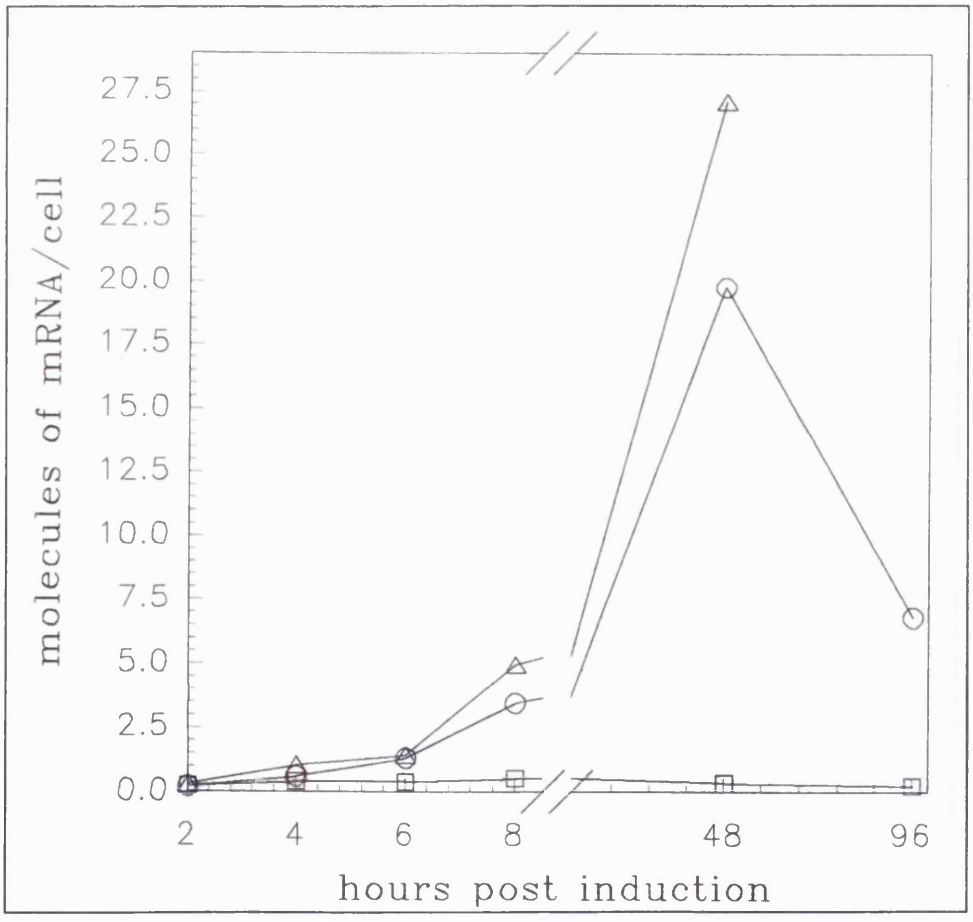


Fig. 3.14 Comparison of inducer effect on the kinetics of induction of CYP2B1 and 2B2 mRNAs. (Δ) picrotoxin, (O) phenobarbital, (□) no inducer.

3.1.4 Expression of CYP2B1/2 proteins

To determine whether the observed increases in CYP2B mRNAs were reflected in corresponding increases in CYP2B proteins we employed the immunoblotting technique to examine the expression and induction by phenobarbital (Fig. 3.15) and by picrotoxin (Fig. 3.16) of these proteins in cultured primary rat hepatocytes. As already mentioned in section 1.2.1.1, CYP2B1 and CYP2B2 proteins only differ by 14 amino acids. Hence, a polyclonal antibody raised against either of the two proteins will detect both proteins. Both compounds were able to strongly induce the expression of CYP2B proteins. Unfortunately it was not possible to accurately measure the level of expression in control samples. For this reason it was only possible to estimate that induction of CYP2B proteins by phenobarbital is at least 20-fold, whereas induction by picrotoxin is at least 11-fold. It was interesting to observe that a protein different from CYP2B was immunoreactive to CYP2B anti-serum (Fig. 3.15, tracks C84, P48, C96). This observation has already been made in the course of other studies (Schuetz et al., 1988; Emi et al., 1990; Sinclair et al., 1991). It is thought that this protein is CYP2C7 on the basis of knowledge derived from studies of immunochemical reactivities of purified CYP proteins with polyclonal antibodies (Ryan & Levin, 1990) and from its mobility after SDS-PAGE. This protein is only 50 % similar in amino acid sequence to CYP2B1/2 but must share some very similar antigenic epitopes. The gene coding for this protein is transcriptionally activated at the onset of puberty and its mRNA reaches a steady-state level in adult females that is 2-fold higher than in adult males (Gonzalez et al., 1986). It is possible therefore that pituitary hormones interfere with CYP2C7 expression in rat and the absence of these hormones in culture allows its expression. Surprisingly, the expression of CYP2C7 was reduced in cells cultured for 48 hr in the presence of PB (Fig. 3.15, track P48) and was undetectable in cells treated for 96 hr with PB (Fig. 3.15, track P96). In contrast picrotoxin did not appear to affect CYP2C7 expression (Fig. 3.16, track c). To our

knowledge, data regarding the effect of phenobarbital on the expression of CYP2C7 have not been published. It should be interesting to investigate the mechanism through which phenobarbital downregulates the expression of this protein.

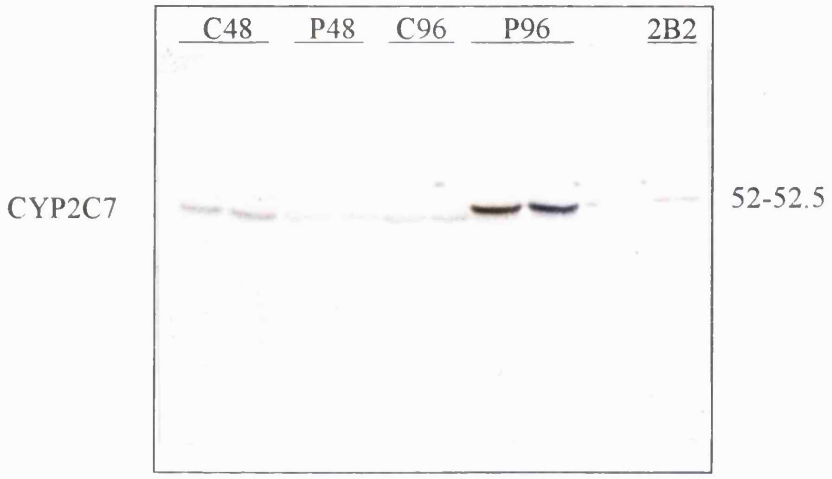


Fig. 3.15 Immunoblot analysis of 20 μg of total cell homogenates isolated from rat hepatocytes cultured for 48 (C48, P48) and 96 hr (C96, P96) in the absence (C48, C96) or presence of PB (P48, P96). Samples are in duplicate. Track 2B2 shows 0.5 ng of purified CYP2B2 protein. Proteins were electrophoresed through a gel containing 10 % acrylamide and CYP2B protein was detected using an anti-CYP2B1/2 serum. The relative molecular masses (x 1000) of CYP2B2 and CYP2B1 are indicated on the right of the figure.

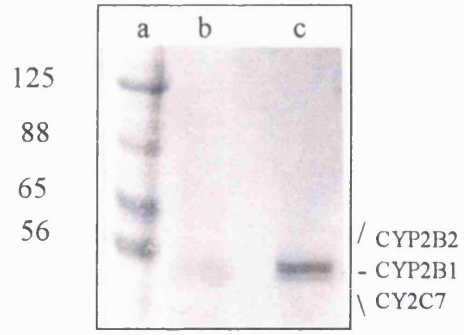


Fig. 3.16 Immunoblot analysis of 20 μg of total cell homogenates isolated from rat hepatocytes cultured in the presence (c) or absence (b) of picrotoxin. Picrotoxin (0.5 mM) was added to the cells 48 hr after attachment and the cells were harvested after a total culture period of 96 hr. Track a shows molecular mass standards with the corresponding relative molecular masses (x 1000) indicated on the left of the figure. Proteins were electrophoresed through a gel containing 7.5 % acrylamide and CYP2B protein was detected using an anti-CYP2B1/2 serum.

3.2 Established hepatoma cells: FAZA 967

Having established culture conditions that allow the expression and induction of CYP2B1/2 mRNAs and proteins in primary rat hepatocytes, we wanted to investigate the possibility of applying these culture conditions to established cell lines. Such culture systems have several advantages over a primary hepatocyte cells system: a) transformed cells are more stable in culture and allow long-term studies, b) primary hepatocyte isolation procedures are tedious and expensive, c) primary hepatocyte cultures are rarely 100 % homogeneous d) transformed cells can be grown in very large numbers and therefore allow more complex studies to be performed. We decided to concentrate our efforts on one cell line, the FAZA 967 cells, since from the literature they appeared to express many of the functions of the adult rat liver (Deschatrette & Weiss, 1974).

From a bile-secreting hepatocellular carcinoma isolated by Reuber in 1961 (Reuber, 1961), Pitot et al. (1964), derived a clonal cell line, H4IIEC3, whose cells express many functions of the hepatocytes of the adult rat (Pitot et al., 1964). Mary Weiss and co-workers (Deschatrette & Weiss, 1974) which were studying this cell line in the early seventies, from time to time, found in the cultures cells that differed in morphology from the original cells. They then went on to isolate and subclone these cells. In particular, FAZA 9, from which the FAZA 967 is derived (a schematic representation of the descent of FAZA 967 cells is shown in Fig. 3.17), were isolated by virtue of the fact that they were resistant to 12 μg/ ml of 8-azaguanine, a known immortalising agent. Their morphology (Fig. 3.18) closely resembles that of adult rat primary hepatocytes, with a polygonal shape, and a large, well-defined round nucleus. They are also characterised by the presence of bile canaliculi. These cells express many of the functions characteristic of well-differentiated adult rat hepatocytes , such as albumin, aldolase, fructose disphosphatase and phosphoenolpyruvate carboxykinase,

the latter two indispensable for the anabolic activity characteristic of hepatocytes. They also retained inducibility by hormones of the two aminotransferases Tyrosine aminotransferase and Alanine aminotransferase (Deschatrette & Weiss, 1974).

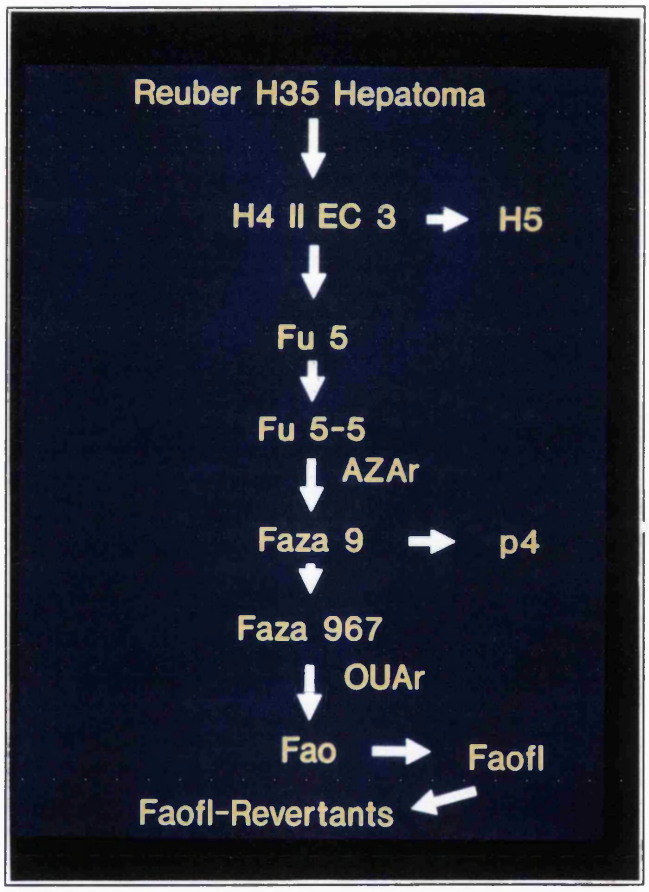


Fig. 3.17 Schematic representation of the clonal derivation of FAZA 967 cells.

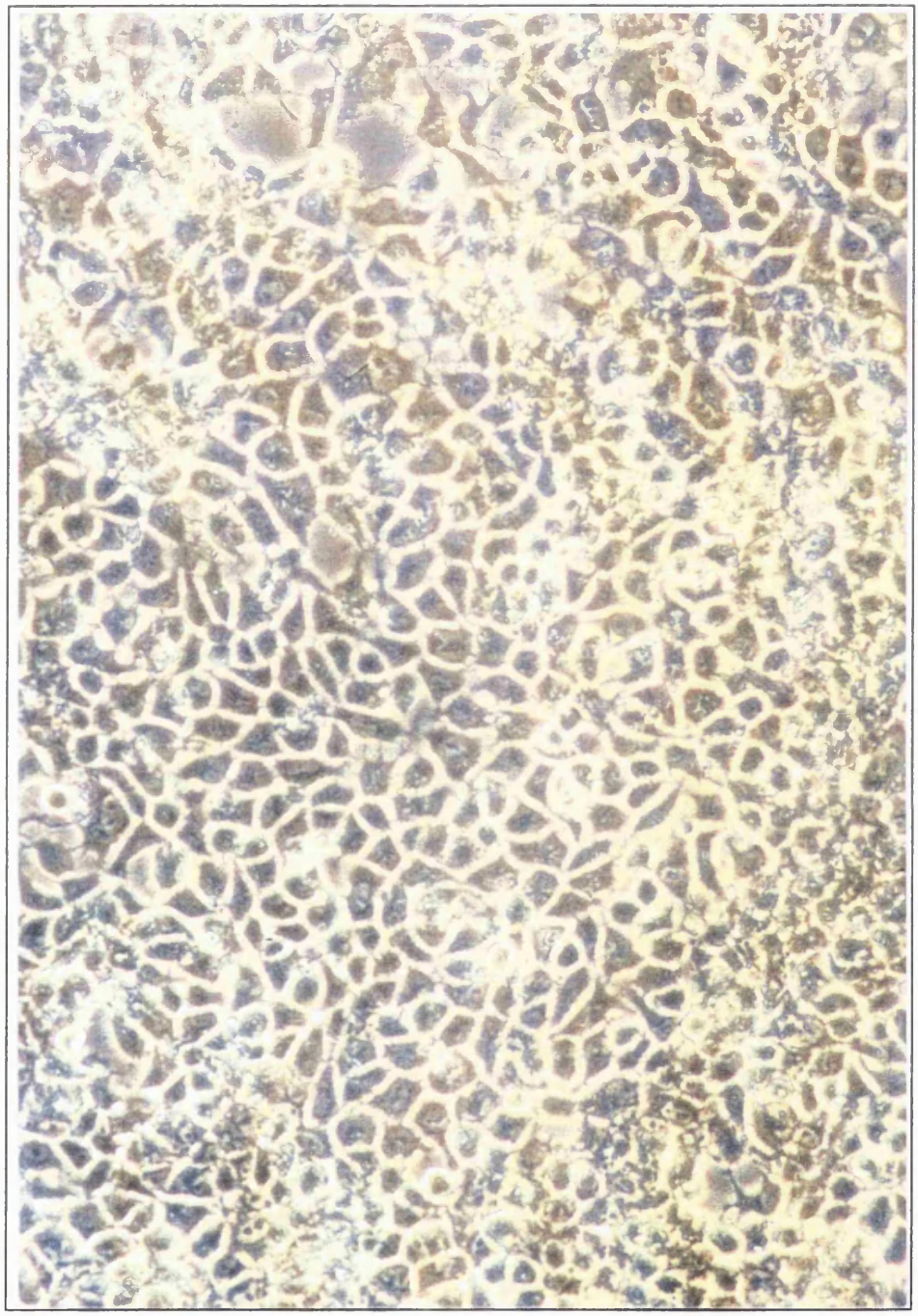


Fig. 3.18 Light photomicrograph of FAZA 967 cells plated on vitrogen-coated Permanox plates (original magnification x 40).

3.2.1 Expression and induction of CYP2B1/2 mRNAs

Initially, we cultured FAZA 967 cells under different conditions and monitored the expression and induction of CYP2B1/2 mRNAs (Fig. 3.19). RNase protection assays showed that best expression and induction levels of CYP2B1/2 mRNAs were achieved when cells were cultured under the same conditions developed for primary hepatocytes (Fig. 3.19, tracks e-h). CYP2B1/2 mRNAs were undetectable in FAZA 967 cells when cultured either on uncoated Nunc plates or on uncoated Permanox plates in the presence of Modified Eagle's Medium (MEM), (Fig 3.19, tracks a-d and i-l). It was interesting to note that good expression and induction levels were achieved in cells cultured on Vitrogen-coated Primaria plates, even in the presence of MEM (Fig. 3.19, tracks m-p), suggesting that the expression and induction of CYP2B1/2 mRNAs in FAZA 967 cells is strongly dependent on the presence of an extracellular matrix. In this respect, these cells differ from primary hepatocytes, since previous reports (Waxman et al., 1990; Sinclair et al., 1990; Sidhu et al., 1993) have shown that the presence of a rich medium such as William's E or Chee's Modified Eagles Medium (CMEM) is the factor that contributes the most to the expression of *CYP2B* genes.

Since the highest level of induction was achieved when FAZA 967 cells were plated on Vitrogen-coated Permanox plates in the presence of William's E medium (Fig. 3.19, track h), we decided to culture cells under these conditions in subsequent experiments. The data obtained from densitometric analysis of autoradiograms from several RNase protection assays, an example of which is shown in Fig. 3.20 (panel A), were analysed statistically and represented graphically in Fig. 3.20, panel B. These data show that these culture conditions consistently support a good induction of CYP2B1/2 mRNAs (approx. 12-fold) after 96 hr of culture in the presence of PB. However, the maximum mean number of CYP2B1/2 mRNA molecules expressed (1.49) is only equal to about 10 % of that expressed in primary

hepatocytes. It is also interesting to note that the highest induction response is observable after 96 hr of culture and not after 48 hr as is the case for primary hepatocytes. This might be due to the fact that in order to increase the number of cells to reasonable levels before being plated under test conditions, FAZA 967 cells are cultured under different conditions (section 2.3). It is possible therefore that the cells may require a few days to acclimatise to the new culture conditions.

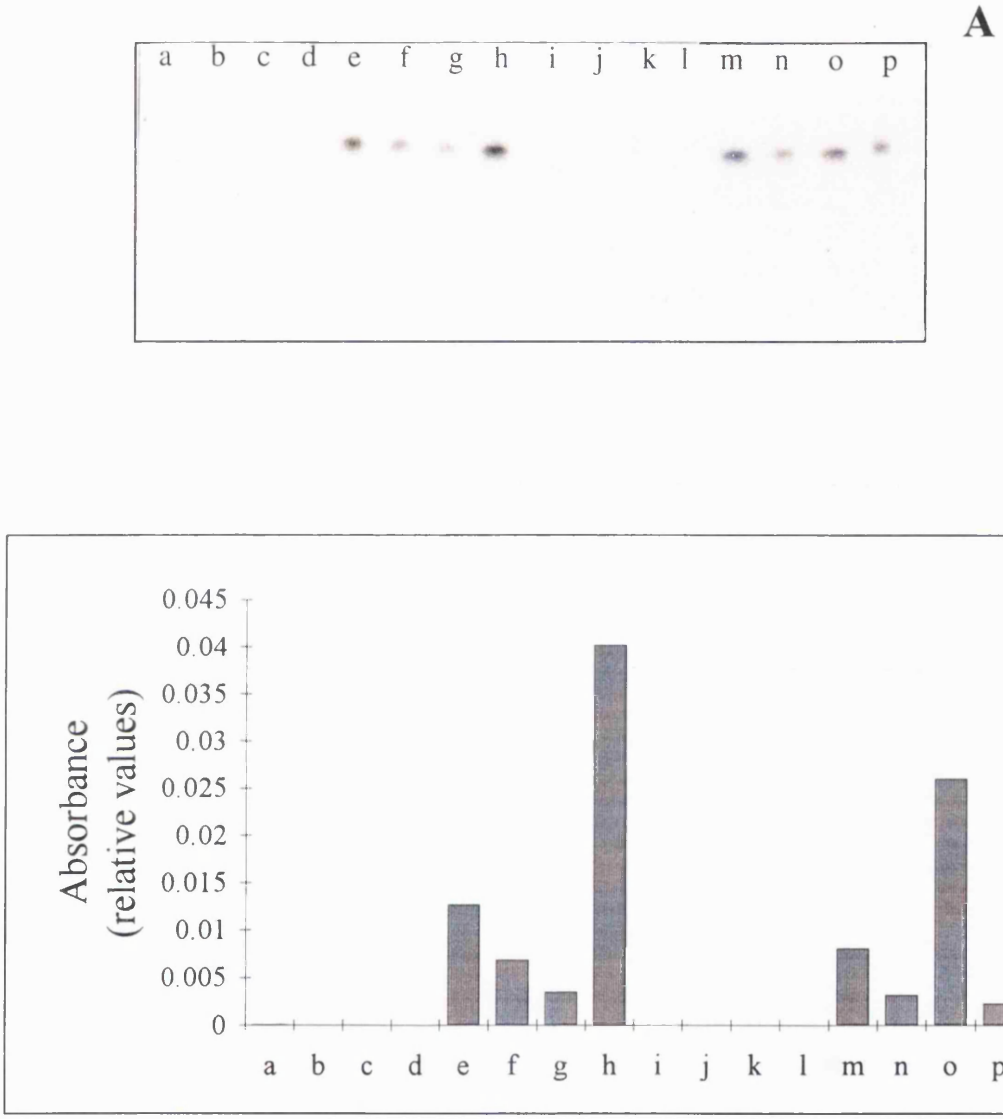


Fig. 3.19 (**A**) RNase protection assay of CYP2B1/2 mRNAs. Total RNA (10 μg) was isolated from FAZA967 cells plated under different culture conditions: (a-d) cells cultured on uncoated Nunc plates in the presence of Modified Eagle's Medium (MEM); (e-h) cells cultured on Vitrogen-coated Permanox plates in the presence of William's E medium; (i-l) cells cultured on uncoated Primaria plates in the presence of MEM; (m-p) cells cultured on Vitrogen-coated Primaria plates in the presence of MEM. The media of cells in tracks b, d, f, h, j, l, n, p was supplemented with 0.75 mM PB. Cells were harvested after 48 hr (a, b, e, f, i, j, m, n) and 96 hr (c, d, g, h, k, l, o, p) of culture under the conditions described above. (**B**) Graphical representation of the results shown in panel A.

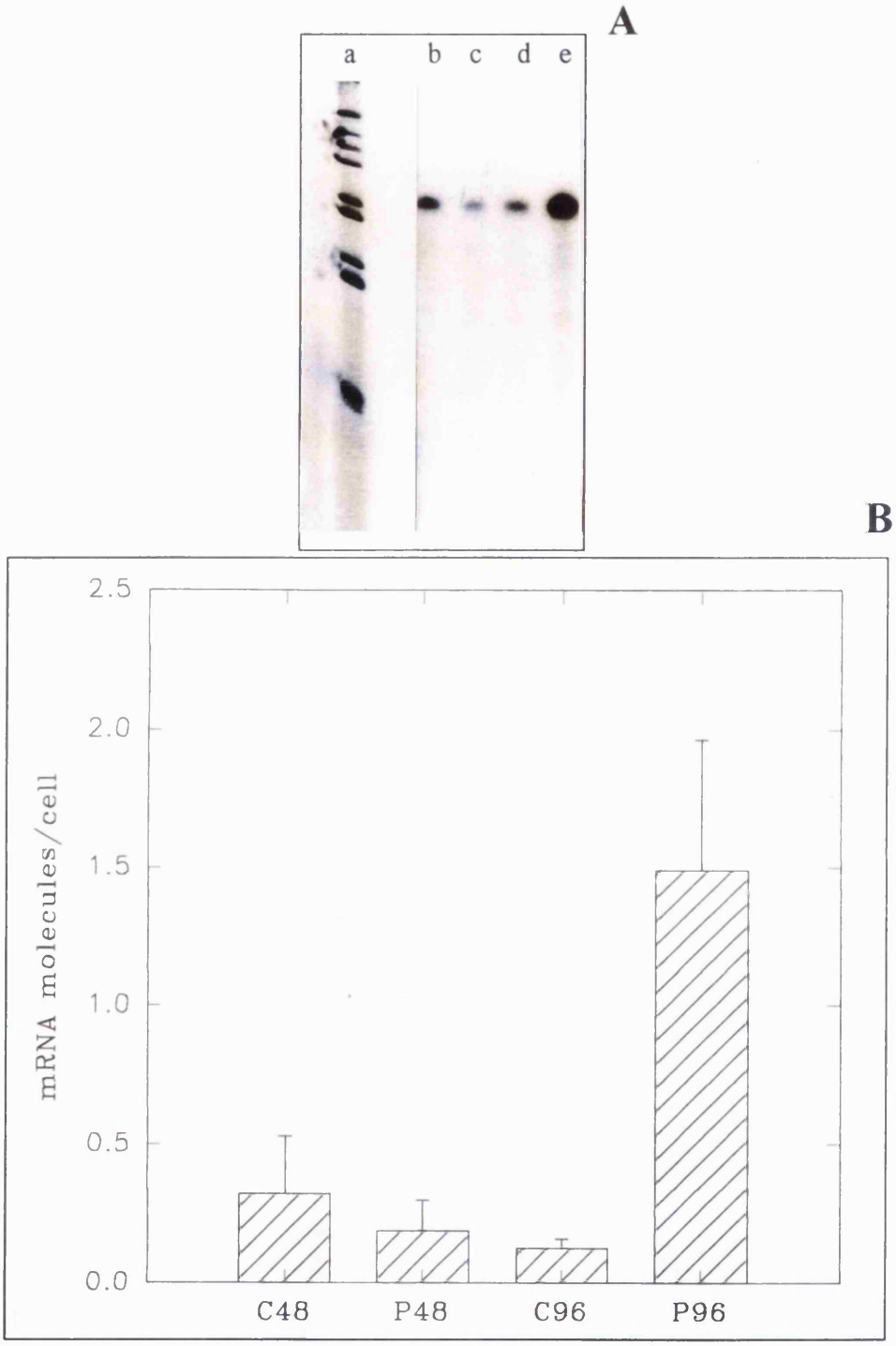


Fig. 3.20 (**A**) Example of an RNase protection of CYP2B1/2 mRNAs in FAZA 967 cells cultured on Vitrogen-coated Permanox plates in the presence of William's E medium. Total RNA (20 μg) was isolated from cells cultured for 48 hr (b, c) and 96 hr (d, e), either in the absence (b, d) or in the presence (c, e) of PB. Track a shows molecular mass standards (1 kb ladder, Bethesda Research Laboratories); (**B**) Graphical representation of the results of several RNase protection assays of CYP2B1/2 mRNAs in FAZA 967 cells. Cells were cultured for 48 hr (C48, P48) and 96 hr (C96, P96) in the absence (C48, C96) or presence (P48, P96) of PB. Filled bars represent mean values (± SD, n=3). $P < 0.05$. (C96 versus P96)

Having established that the culture conditions developed for primary hepatocytes support induction by PB of CYP2B1/2 mRNAs also in FAZA 967 cells, we went on to investigate whether these conditions allow induction by picrotoxin. Indeed, RNase protection assays (Fig. 3.21) showed that these culture conditions support a strong induction by picrotoxin of CYP2B1/2 mRNAs (≈14-fold; Fig. 3.21, panel B). Interestingly, the highest response was observed 48 hr after addition of the inducer (track f) and declined within the next 48 hr (track h). Moreover, picrotoxin appeared to stimulate the expression of CYP2B1/2 mRNAs to a level that is 1.4- to 2-fold higher than that achieved in the presence of PB (≈3.4 molecules for picrotoxin compared with ≈1.9 molecules for PB). It appears therefore that picrotoxin is more potent than phenobarbital at stimulating the expression of CYP2B1/2 genes. The difference observed in the time course of induction by picrotoxin and phenobarbital of CYP2B mRNAs may suggest that, at least in this cell system, the two compounds could mediate this induction via different mechanisms. However, this observation may also be a consequence of the fact that picrotoxin could be unstable in an aqueous solution. Because of the very low solubility of picrotoxin in water, we could not prepare concentrated stock solutions of this compound. Therefore, in these experiments, the appropriate amounts of picrotoxin were dissolved directly into 500 ml of medium, which was then used throughout the experiment. It is possible therefore that picrotoxin was not stable in this solution for longer than 48 hr. Unfortunately there are no data available regarding the stability of picrotoxin in aqueous solutions (Sigma Chemical Co., personal communication).

In a simultaneous control experiment, in which cells were cultured on uncoated Nunc plates in the presence of MEM medium (Fig 3.21, tracks a-d), CYP2B1/2 mRNAs were virtually undetectable in total RNA isolated from both untreated (tracks a, c) and picrotoxin-treated (tracks b, d) cells.

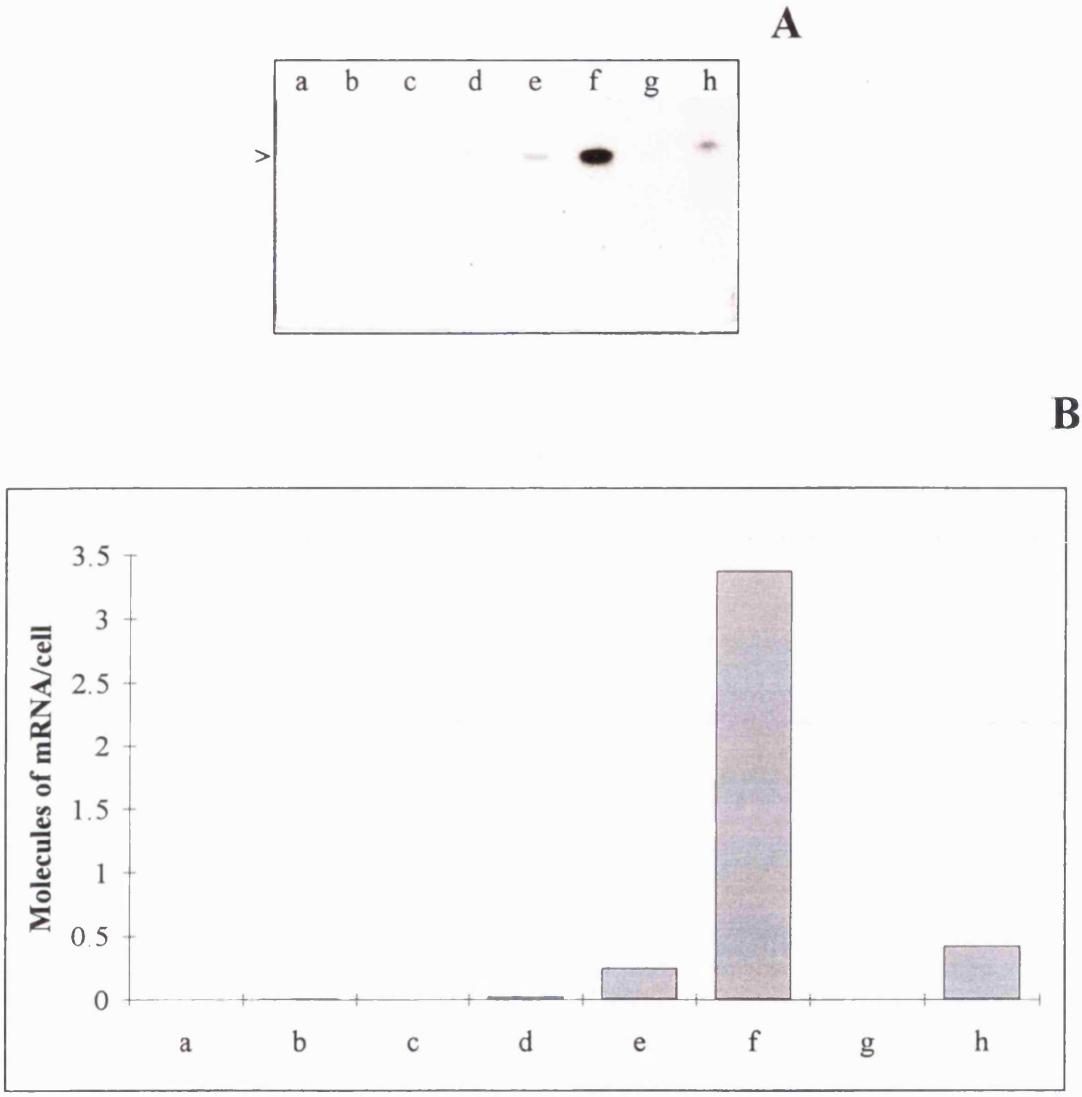


Fig. 3.21 Effect of picrotoxin on the expression of CYP2B1/2 mRNAs in FAZA 967 cells. (**A**) RNase protection of CYP2B1/2 mRNAs in total RNA (20 μg) isolated from cells cultured either on uncoated Nunc plates in the presence of MEM (a-d), or on Vitrogen-coated Permanox plates in the presence of William's E medium (e-h). Untreated- (a, c, e, g,) or picrotoxin-treated (b, d, f, h) cells were harvested after either 48 hr (a, b , e, f) or 96 hr (c, d, g, h) of culture. The arrowhead indicates the fully-protected fragments. (**B**) Graphical representation of the results shown in panel A.

3.2.2 Expression of CYP2B1/2 proteins

We employed the immunoblotting technique to detect CYP2B1/2 proteins in FAZA 967 cells. Protocols employing horse-radish-peroxidase- or alkaline-phosphatase-conjugated secondary antibodies were tried. Unfortunately, although several attempts were made no CYP2B1/2 proteins were detected. It is doubtful that this observation results from the fact that in FAZA 967 cells CYP2B1/2 mRNAs are translation-incompetent. Almost certainly the immunoblotting protocols employed were not sensitive enough to detect CYP2B1/2 proteins at the very low concentration at which these proteins are expected to be present (CYP2B1/2 mRNA levels in PB-treated FAZA 967 cells were only about 10 % of those observed in PB-treated primary hepatocytes). It is therefore possible that CYP2B1/2 proteins are expressed and induced by picrotoxin and phenobarbital in the FAZA 967 cells, however a more sensitive assay is required to detect them.

3.3 Expression of other drug-metabolising enzymes

To ascertain the overall competence of the cell systems developed as *in vitro systems* suitable for the study of drug metabolism, we monitored the expression of several other drug-metabolising enzymes. In particular we looked at the expression of P450 reductase, FMO1 and glutathione-S-transferases.

3.3.1 Expression of P450 reductase

Expression of P450 reductase is essential for all the reactions catalysed by CYP enzymes of the endoplasm reticulum. Using a rabbit anti-rat-P450-reductase serum and immunoblotting, we monitored the expression of P450 reductase both in primary hepatocytes (Fig. 3.22, tracks b-i) and in FAZA 967 cells (Fig. 3.22, tracks j-m). The effect of inducers on the expression of this protein was also investigated. Primary hepatocytes expressed P450 reductase at high levels, whether cultured on uncoated (Fig. 3.22, tracks f and g) or Vitrogen-coated (Fig. 3.22, tracks b-e) Permanox plates. Addition of PB (Fig. 3.21, tracks b, e, and g) resulted in an increase in expression that was 1.7- to 3-fold higher than that observed in untreated cultures (Fig. 3.22, tracks c, d, and f). These observations are consistent with those made during the course of studies that have monitored expression of P450 reductase *in vivo* (Shephard et al., 1983) and *in vitro* (Waxman et al., 1990; Akrawi et al., 1993). Surprisingly, when cells were cultured in the presence of picrotoxin (Fig. 3.22, track h), P450 reductase expression increased only 1-fold or less above levels observed in control cultures (Fig. 3.22, track i). Because both picrotoxin and phenobarbital strongly stimulate CYP2B1/2 expression, this observation may suggest that the induction by phenobarbital of P450 reductase expression operates via a mechanism that is different from that involved in the stimulation of CYP2B1/2 expression.

P450 reductase expression could also be observed in FAZA 967 cells (Fig. 3.22,

tracks j-m). In these cells, phenobarbital (Fig. 3.22, tracks k and m) stimulated expression of P450 reductase to levels 8- to 10-fold higher than those observed in control cultures (Fig. 3.22, tracks j and l). However, overall expression levels were significantly lower than those observed in primary hepatocytes.

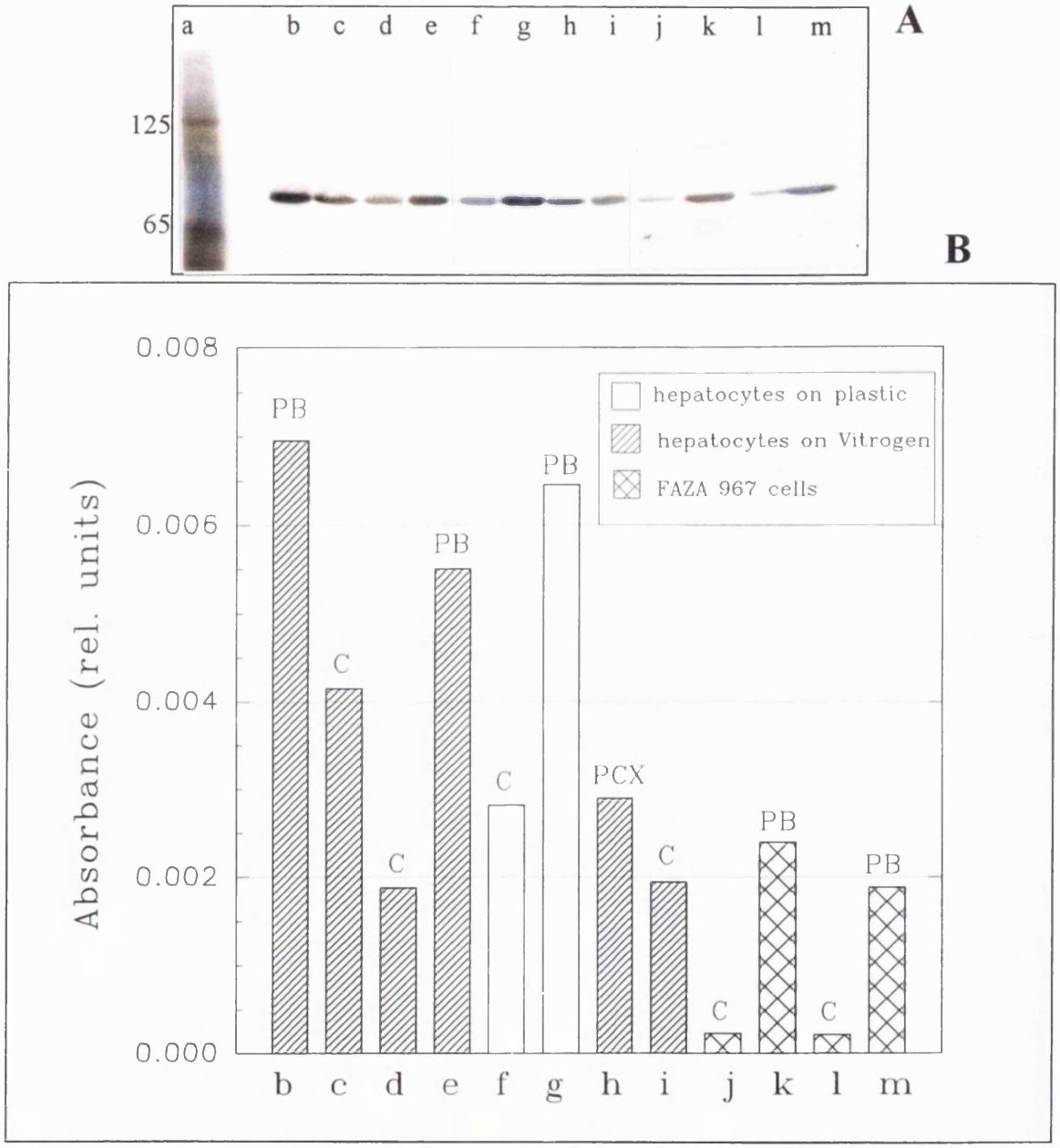


Fig. 3.22 Expression of P450 reductase. (**A**) Immunoblot analysis of 20 μg of total cell homogenates isolated from either primary rat hepatocytes (tracks b-i), or from FAZA 967 cells (j-m). All cells were cultured on Vitrogen-coated Permanox plates, in the presence of William's E medium. The only exception is shown in tracks f and g where homogenates were isolated from primary hepatocytes cultured on uncoated Permanox plates. Cells were kept in culture for 48 hr (b, c, f-k) and 96 hr (d, e, l, m) either in the presence of PB (b, e, g, k, m) or PCX (h), or in the absence of inducer (c, d, f, i, j, l). (**B**) Graphical representation of the results shown in **A**.

3.3.2 Expression of FMO1

In order to ascertain the potential of the cell systems investigated so far to be used as model systems for the study of FMO enzymes, we decided to monitor the expression of FMO1. This enzyme has been found to be expressed at various levels in the liver of several mammals, including pig (Ziegler & Mitchell, 1972; Gasser et al., 1990), rat (Kimura et al., 1983), mouse (Sabourin et al., 1984), and rabbit (Tynes & Hodgson, 1985).

Because FMO orthologues share extensive amino acid sequence identity (>82 %; Lawton & Philpot, 1993), we performed immunoblot analysis of total cell homogenates (Fig. 3.23) using a rabbit polyclonal antibody raised against pig FMO1 (a gift from Dr. D. M. Ziegler, Clayton Foundation Biochemical Institute, University of Texas, Texas, U.S.A.). FMO1 expression was detected in both primary hepatocytes (Fig. 3.23, tracks b-g) and FAZA 967 cells (Fig. 3.23, tracks h-k). In none of the cell systems investigated [primary hepatocytes plated on Vitrogen-coated (Fig. 3.23, tracks b-e) or uncoated (Fig. 3.23, tracks f and g) Permanox plates, and FAZA 967 cells plated on Vitrogen-coated Permanox plates (Fig. 3.23, tracks h-k)] did phenobarbital (Fig. 3.23, tracks c, e, g, i, and k) cause any significant change in FMO1 expression, when compared with expression in untreated cultures (Fig. 3.23, tracks b, d, f, h, and j). Overall expression was best in primary hepatocytes cultured on Vitrogen-coated Permanox plates (Fig. 3.23, tracks b-e), with highest expression detected in cells that were kept in culture for 48 hr (Fig. 3.23, tracks b and c). A decline in FMO expression was however evident in these cells when kept in culture for a further 48 hr (tracks d and e). Levels of FMO1 expression were comparable in primary hepatocytes plated on uncoated Permanox plates (Fig. 3.23, tracks f and g) and FAZA 967 cells (Fig. 3.23, tracks h-k). In the latter cell system, there was no apparent difference in expression levels between cells cultured for 48 hr (Fig. 3.23, tracks h and i) and cells cultured for 96 hr (Fig. 3.23, tracks j and k).

The defined culture conditions developed during the course of this study can therefore sustain high levels of FMO1 expression in primary hepatocytes and a constant basal level of expression in FAZA 967 cells. Hence, these results indicate it may be possible to use these cell systems as a tool for investigating the regulation and expression of FMO enzymes under carefully controlled conditions. Provided it is possible to cope with lower expression levels, FAZA 967 cells may be the system of choice because of the obvious advantages associated with an established cell line. Obviously, it remains to be established whether the FMO1 protein expressed in both cell systems is active.

Finally, in agreement with previous studies both *in vivo* (Ziegler et al., 1980) and *in vitro* (Coecke et al., 1993), our results indicate that PB does not increase FMO1 expression. This observation is a good indication that, in the culture systems developed in the course of this study, phenobarbital does not cause a general increase in the expression of all proteins, but specifically stimulates the expression of some drug-metabolising enzymes.

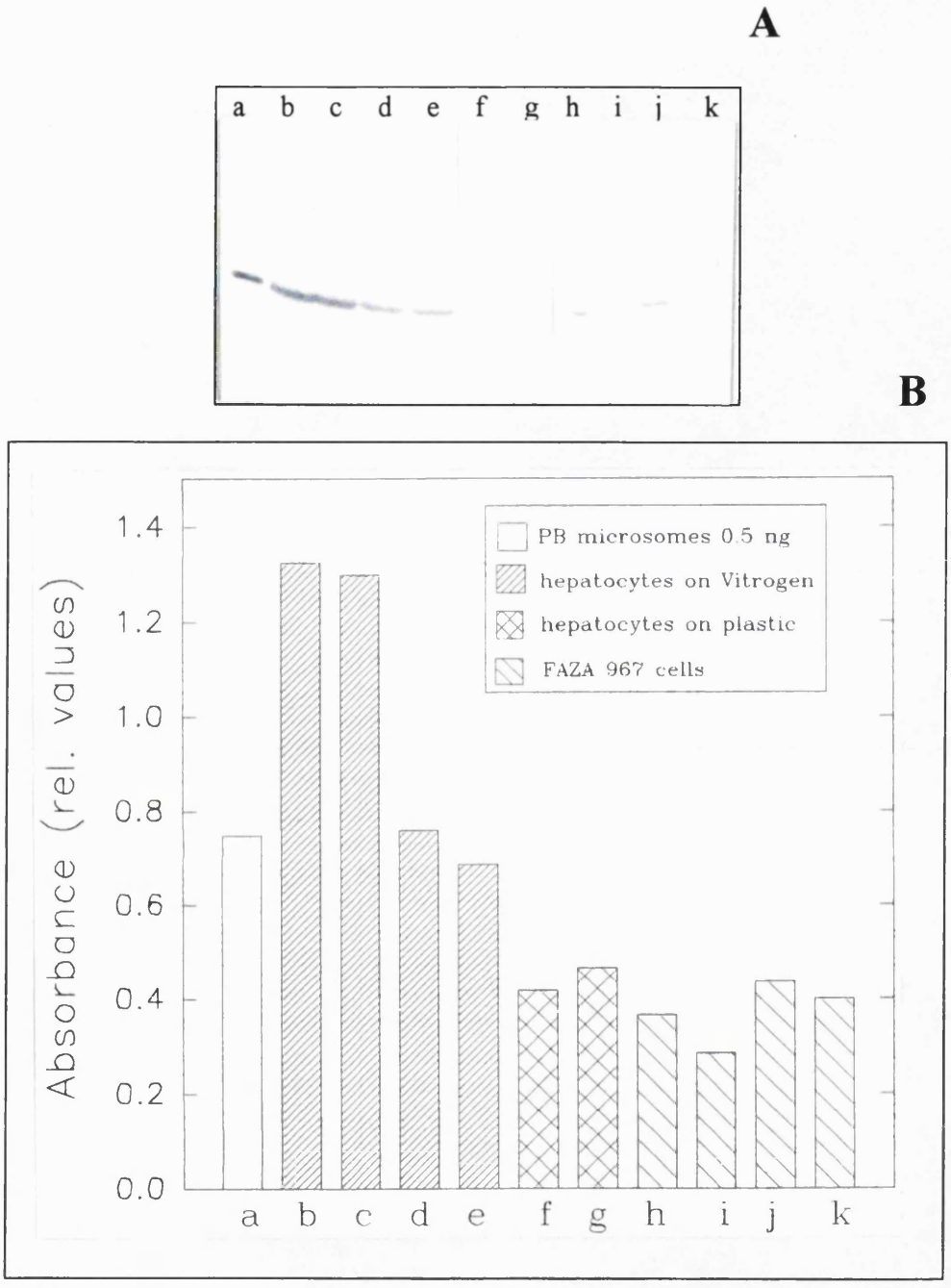


Fig. 3.23 Expression of FMO1. (**A**) Immunoblot analysis with a rabbit polyclonal antibody raised against pig FMO1. All samples were electrophoresed through a 10 % SDS polyacrylamide gel. Total cell homogenates (20 μg) were isolated from primary hepatocytes (tracks b-g) and FAZA 967 cells (h-k) cultured for 48 hr (tracks b, c, f-i) or 96 hr (d, e, j, k) either in the absence (b, d, f, h, j) or presence (c, e, g, i, k) of PB. Homogenates in tracks f and g were isolated from primary hepatocytes cultured on uncoated Permanox plates, those in tracks b-e, and h-k from cells cultured on Vitrogen-coated Permanox plates. Track a shows 0.5 ng of microsomes isolated from the liver of a PB-treated rat. (**B**) Graphical representation of the results shown in panel **A**.

3.3.3 Expression of Glutathione S-transferases: detection of GST1-1/2-2, GST3-3/4-4, and GST7-7

Having established that the cell systems developed during the course of this study supported the expression of several phase I drug-metabolising enzymes, we now wanted to test whether they could also express phase II enzymes. As it is not possible to monitor all enzymes belonging to this group, we decided to concentrate on glutathione S-transferases mainly for two considerations. In addition to the intrinsic interest of GSTs as the largest family of phase II drug-metabolising enzymes, the expression of these enzymes, like that of CYP2B1/2 proteins, is difficult to preserve in primary hepatocytes cultured conventionally. For example, it was shown that the expression of GSTs belonging to the α class decreases, whereas that of class μ and π is markedly increased (Abramovitz et al., 1989; Vandenberghe et al., 1989; Vandenberghe et al., 1992). This pattern of GST expression is indicative of a general state of foetalization of cultured hepatocytes (Tee et al., 1992). In particular, GST7-7 (the only GST of the π class in rat) is absent from normal adult rat hepatocytes whereas it is expressed in foetal hepatocytes (Tee et al., 1992) or specifically induced at an early stage of chemical hepatocarcinogenesis (Kitahara et al., 1984; Satoh et al., 1985). Hence, data on the expression of GSTs belonging to the α, μ, and π classes would not only provide us with valuable information regarding the capacity of our cell systems to express phase II enzymes, but it would provide also information on the general state of differentiation the cells are in. We used the immunoblotting technique to monitor the expression of GST2-2/1-1 (α class, Fig. 3.24), GST3-3/4-4 (μ class, Fig. 3.25) and GST7-7 (π class, Fig. 3.26) in both primary hepatocytes and FAZA 967 cells. Proteins were detected using rabbit anti rat GST2-2, 3-3, and 7-7 sera (Biotrin International, Ireland).

3.3.3.1 Expression of GST2-2/1-1

The anti GST2-2 serum used in the course of these experiments cross-reacts with GST1-1 (Biotrin International technical bulletin) and hence the results shown in Fig. 3.24 represent the expression of both GST2-2 and GST1-1. Expression of these proteins (Fig. 3.24) was detected in all cell systems investigated, which include primary hepatocytes cultured on uncoated (Fig. 3.24, tracks f and g) or Vitrogen-coated (Fig. 3.24, tracks b-e, h and i) Permanox plates, and FAZA 967 cells (Fig. 3.24, tracks j-m). In agreement with previously published observations (Vandenberghe et al., 1988) overall GST2-2/1-1 expression levels were lower in primary hepatocytes cultured for 96 hr (Fig. 3.24, tracks d and e) than in cells cultured for 48 hr (Fig. 3.24, tracks b and c). When culture media were supplemented with 0.75 mM PB, in both primary hepatocytes cultured on uncoated (Fig. 3.24, track g) and Vitrogen-coated (Fig. 3.24, tracks b, and e) Permanox plates, expression of GST2-2/1-1 was 1.3- to 2-fold higher than that observed in their respective control cultures (Fig. 3.24, track f, and tracks c and d). The level of GST2-2/1-1 induction observed in these cells is similar to induction levels achieved during the course of a previous study (Vandenberghe et al., 1989). In addition to PB, we also found picrotoxin capable of stimulating GST2-2/1-1 expression in primary hepatocytes. When cells were cultured for 48 hr in the presence of 0.5 mM picrotoxin (Fig. 3.24, track h), the amounts of GST2-2/1-1 protein were about 2-fold higher than those detected in untreated cultures (Fig. 3.24, track i). To our knowledge, there are no data available in the literature regarding picrotoxin stimulation of GST expression in primary hepatocytes. However, our observation seems to be in broad agreement with measurements of GST activity made *in vivo* during the course of a previous study (Yamada et al., 1993).

FAZA 967 cells cultured in the presence of PB (Fig. 3.24, tracks k and m) also expressed GST2-2/1-1 proteins at levels 1.5- to 3-fold higher than those detected in

untreated cultures (Fig. 3.24, tracks j and l). This observation was not surprising since expression of GST1 subunit has been detected and shown to be inducible by PB in H4IIEC3 and Fao cells (Daniel et al., 1989). As shown in Fig. 3.17, FAZA 967 cells were derived from H4IIEC3 cells, whereas Fao cells were cloned directly from FAZA 967 cells (Deschatrette & Weiss, 1974).

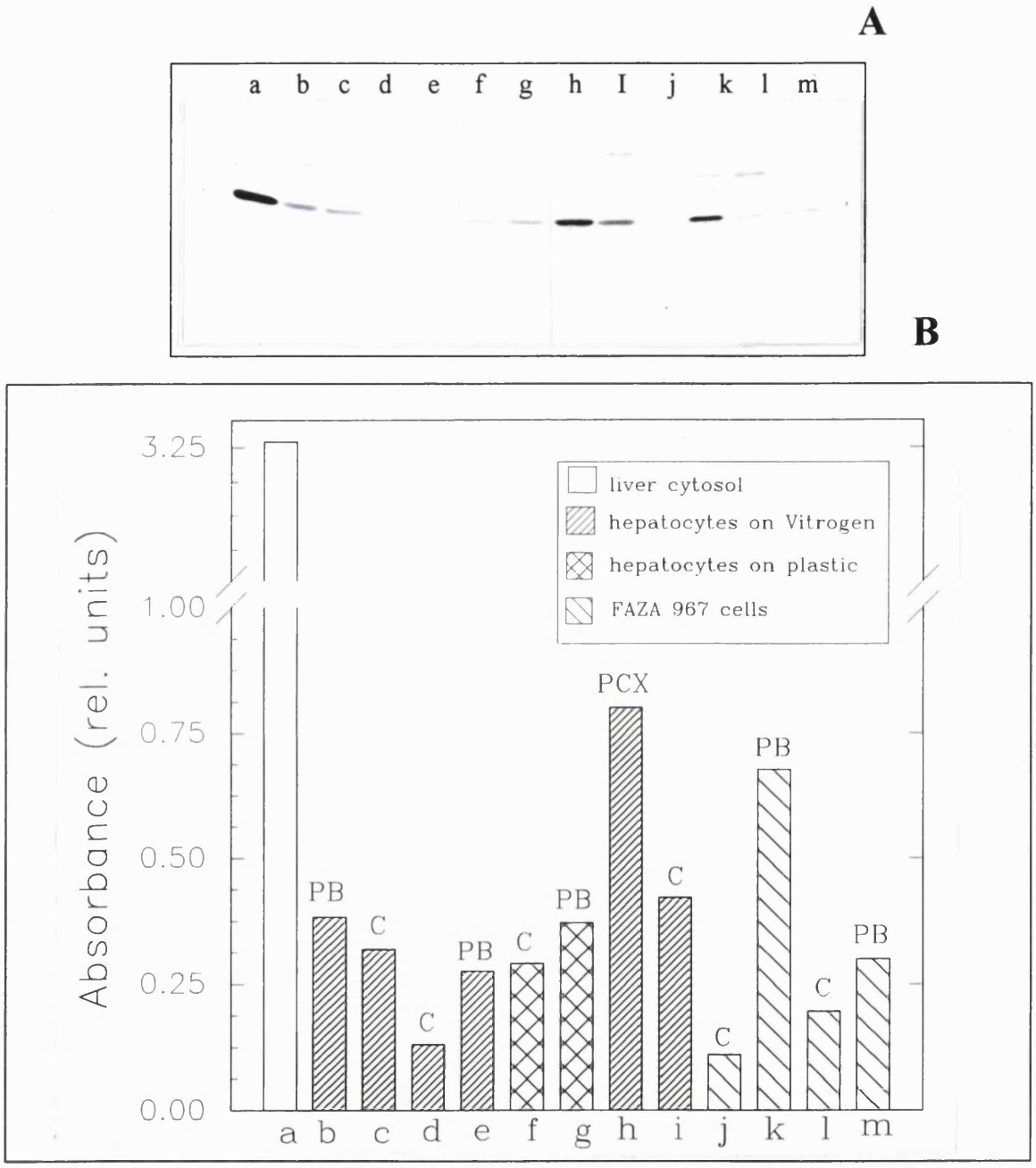


Fig. 3.24 Expression of GST2-2/1-1. (**A**) Immunoblot analysis of 10 μg of either liver cytosolic extracts (track a) or total cell homogenates isolated from cells cultured in the presence of William's E medium on Vitrogen-coated (b-e, h-m) or uncoated (f, g) Permanox plates. Cells were cultured for 48 hr (b, c, f-k) or 96 hr (d, e, l, m) either in the absence of inducer (c, d, f, i, j, l) or in the presence of PB (b, e, g, k, m) or PCX (h). Homogenates in lanes b-i were isolated from primary rat hepatocytes; homogenates in lanes j-m were isolated from FAZA 967 cells. Proteins were separated by means of electrophoresis through a 15 % SDS polyacrylamide gel prior to blotting. (**B**) Graphical representation of the results shown in panel A.

3.3.3.2 Expression of GST3-3/4-4

The anti GST3-3 serum used in the course of these experiments cross-reacts with GST4-4 (Biotrin International technical bulletin). Hence the results shown in Fig. 3.25 represent the expression of both GST3-3 and GST4-4. Expression of GST3-3/4-4 was clearly detectable in primary hepatocytes, whether cultured on Vitrogen-coated (Fig. 3.25, tracks f, g and k-n) or on uncoated (Fig. 3.25, tracks i and j) Permanox plates. In contrast, GST3-3/4-4 expression was almost undetectable in FAZA 967 cells (tracks b-e). Primary hepatocytes cultured on Vitrogen-coated Permanox plates did not express GST3-3/4-4 proteins at levels significantly higher than those observed in primary hepatocytes plated on uncoated Permanox plates. Likewise, basal GST3-3/4-4 expression levels in primary hepatocytes cultured for 96 hr (Fig. 3.25, track l) were not significantly different from basal expression levels detected in primary hepatocytes cultured for 48 hr (Fig. 3.25, track n).

When primary hepatocytes were cultured for 48 hr on Vitrogen-coated Permanox plates in the presence of PB (Fig. 3.25, track m), GST3-3/4-4 expression was increased ≈ 1.5-fold in comparison with untreated cultures (Fig. 3.25, track n). The same induction response was observed in primary hepatocytes plated on uncoated Permanox plates, where expression of GST3-3/4-4 in PB-treated cultures (Fig. 3.25, track j) was about 1.3-fold higher than expression in untreated cultures (Fig. 3.25, track i). However, the inducing effect of PB appeared to be increased when cells were kept in culture for a total period of 96 hr. In this case, expression of GST3-3/4-4 in PB-treated cultures (Fig. 3.25, track k) was ≈ 2.7-fold higher than in untreated cultures (Fig. 3.25, track l). This higher induction response observed in cells kept in culture for longer, is in good agreement with previously published observations (Vandenberghe et al., 1989). A strong induction response was also observed when primary hepatocytes were plated for 48 hr on Vitrogen-coated Permanox plates in the presence of 0.5 mM picrotoxin (PCX). In PCX-treated cells (Fig. 3.25, track f), GST3-3/4-4

expression was ≈2.7-fold higher than in untreated cultures (Fig. 3.25, track g).

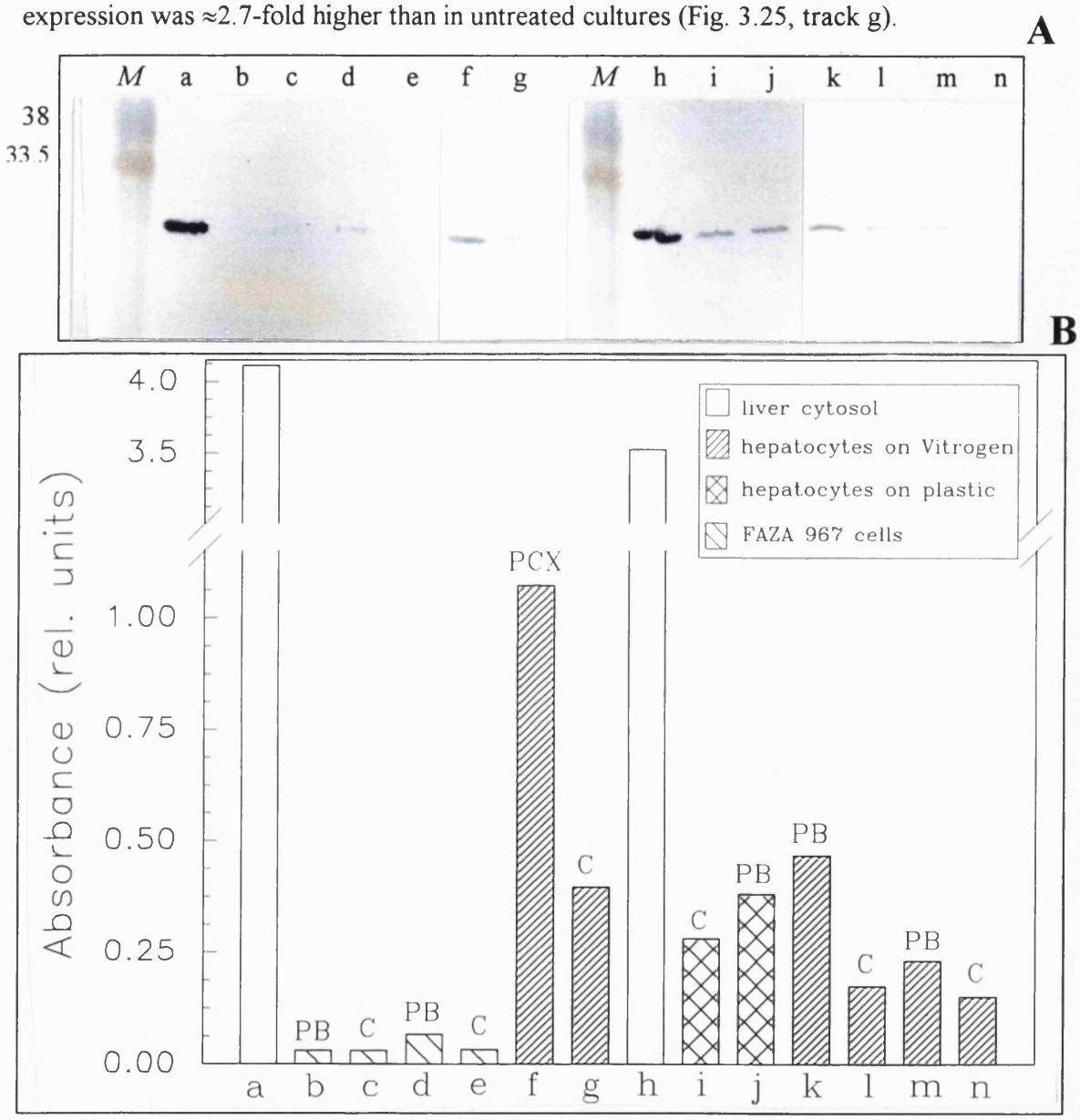


Fig. 3.25 Expression of GST 3-3/4-4. (**A**) Immunoblot analysis of 10 μg of either liver cytosolic extracts (tracks a and h) or total cell homogenates isolated from cells cultured in the presence of William's E medium on Vitrogen-coated (b-g, k-n, f and g) or uncoated (i, j) Permanox plates. Homogenates in tracks f, g and i-n were isolated from primary rat hepatocytes; homogenates in tracks b-e were isolated from FAZA967 cells. Cells were cultured for 48 hr (d, e, f, g, i, j, m, n) or 96 hr (b, c, k, l) either in the absence of inducer (c, e, g, i, l, n) or in the presence of PB (b, d, j, k, m) or PCX (f). Tracks *M* show molecular mass standards with the corresponding relative molecular masses (x 1000) indicated on the left of the figure frame. Proteins were separated by means of electrophoresis through a 15 % SDS polyacrylamide gel prior to blotting. (**B**) Graphical representation of the results shown in panel A. "liver cytosol" in the graph legend indicates liver cytosolic extracts.

3.3.3.3 Expression of GST7-7

Expression of GST7-7 was undetectable in primary hepatocytes cultured for 48 hr (tracks h and i) and 96 hr (tracks f and g) on Vitrogen-coated Permanox plates, either in the absence (tracks g and i) or in the presence of PB (tracks f and h). The same was true also of primary hepatocytes plated on uncoated Permanox plates (data not shown). Expression of GST7-7, as expected (Tee et al., 1992), was also undetectable in liver cytosolic extracts (Fig. 3.26, track a). High levels of GST7-7 expression were however observed in FAZA 967 cells (Fig. 3.26, tracks b-e). When these cells were cultured for 48 hr (tracks d) and 96 hr (track b) in the presence of 0.75 mM PB GST7-7 expression was respectively 3- and 2-fold higher than in untreated cells, kept in culture for the same period of time (Fig. 3.26, 48 hr: track e; 96 hr: track c).

During the course of previous studies, rat hepatocytes in continuous culture were found to express GST7 protein (Vandenberghe et al., 1988) and mRNA (Vandenberghe et al., 1989). Expression of GST7 mRNA was found to be inducible by phenobarbital (Vandenberghe et al., 1989, 1992). Insulin was also shown to promote the accumulation of GST7 mRNA in primary hepatocytes and this effect was blocked by dexamethasone (Abramovitz et al., 1989). More recent data has shown that GST7 mRNA is significantly induced by epidermal growth factor (EGF) or insulin in serum-free primary cultures of rat hepatocytes (Hatayama et al., 1991). Both insulin and dexamethasone are present in our cultures. In agreement with Abramovitz et al. (1989), the lack of GST7-7 expression in our primary hepatocytes system may be due therefore to the presence of dexamethasone, interfering with the positive effect of insulin on GST7-7 expression. Alternatively, the concentration of insulin (1.7 μM) employed in the course of this study is too low to promote GST7-7 expression. Hence, observations made during the course of this study and those

made by Abramovitz et al. (1989) and by Hatayama et al. (1991) suggest that the spontaneous induction of GST7 expression observed in hepatocytes cultures containing serum may be due to the presence of hormones and growth factors in the added serum. This hypothesis seems to be strengthened by the fact that insulin was present serum-free cultures in which GST7 expression was detected (Vandenberghe et al., 1988).

Interestingly, FAZA 967 cells, although cultured under the same chemically-defined conditions as those utilised for primary hepatocytes, expressed high levels of GST7-7. This phenomenon may be due to the fact that these cells, prior to being cultured under chemically-defined conditions, are propagated in media containing serum. However, it is unlikely that the effect of serum lasts throughout the four days during which FAZA 967 cells were cultured under defined conditions. A more likely explanation may be directly connected with the stimulation of GST7 expression by insulin and EGF. Induction by these hormones of GST7 expression in primary hepatocytes was shown to be associated with an enhanced expression of proto-oncogenes *c-jun* and *c-fos* (Hatayama et al., 1991). Increased levels of *c-jun* expression have been associated with hepatocellular carcinomas (Sakai et al., 1989) and, because FAZA 967 are fully-transformed cells that were isolated from a rat hepatocellular carcinoma, it is possible that they already have raised levels of *c-jun.*

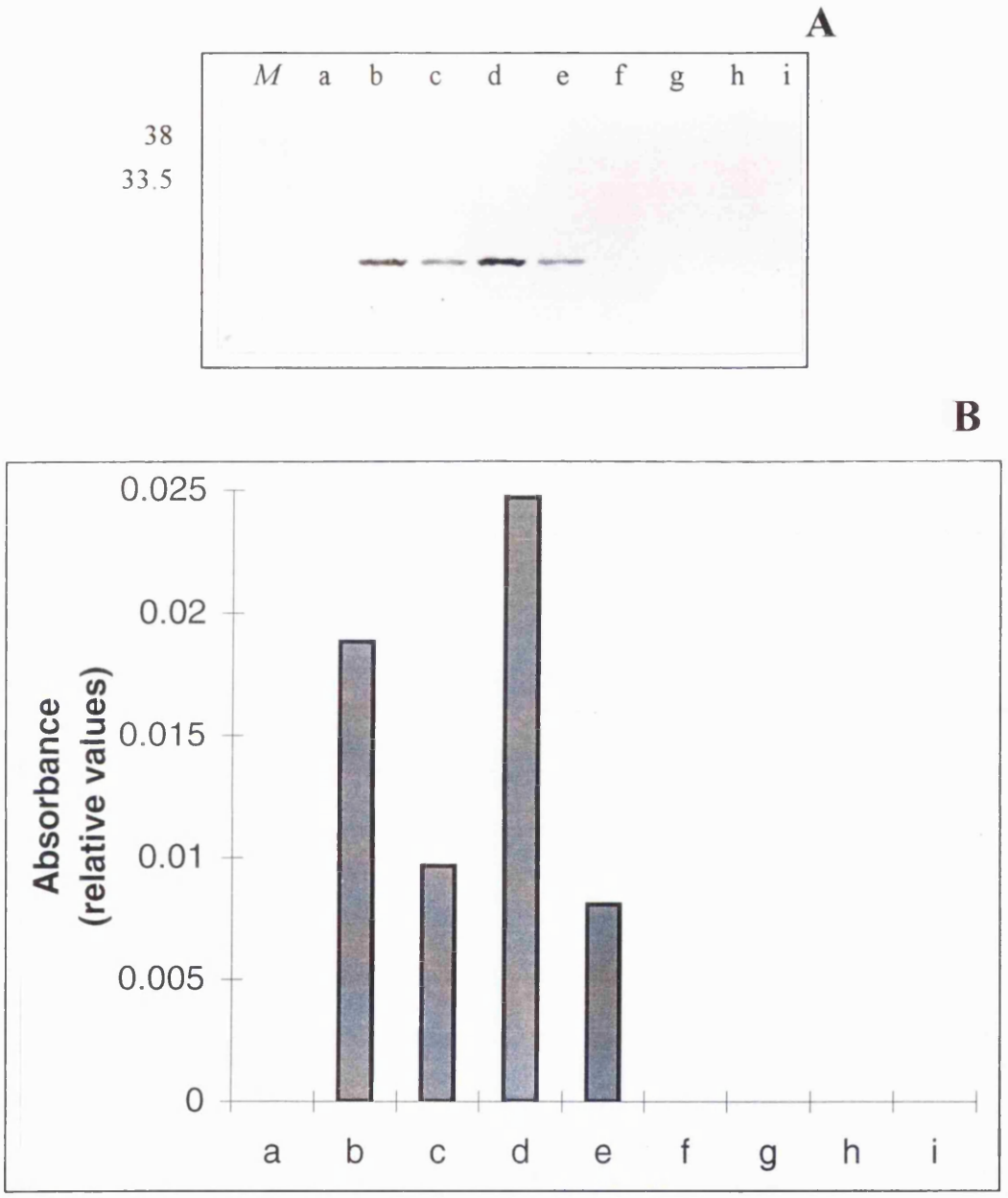


Fig. 3.26 Expression of GST 7-7. (**A**) Immunoblot analysis of 10 μg of either liver cytosolic extracts (track a) or total cell homogenates isolated from cells cultured on Vitrogen-coated Permanox plates in the presence of William's E medium (b-i). Cells were cultured for 48 hr (d, e, h, i) or 96 hr (b, c, f, g) either in the absence (c, e, g, i) or in the presence (b, d, f, h) of PB. Track *M* shows molecular mass standards with the corresponding relative molecular masses (x 1000) indicated on the left of the frame. Proteins were separated by means of electrophoresis through a 15% SDS polyacrylamide gel prior to blotting; (**B**) Graphical representation of the results shown in panel **A**.

3.3.3.4 Considerations on the overall pattern of expression of GST proteins in the cell systems investigated

Unfortunately, due to the lack of purified GST proteins, we could not quantify the expression of GSTs in the cell systems investigated. Indeed, the immunoblot analysis we employed in the course of this study is not very accurate and the more sensitive HPLC-based assay (Vandenberghe et al., 1988, 1989) is required to precisely measure GST subunit concentrations. In addition, because of changes in the titre of antibodies and the different responses of the antibodies to the colour development reaction, it was not possible to compare directly the densitometric results of blots developed at different times. However, we could still make an approximate comparison of the levels of expression of the various GSTs in untreated cultures, by expressing the densitometric results of the cell samples as a percentage of the results of liver extracts, obtained using the same antibody and developed at the same time as the cell samples. It should be emphasised at this point that the results of GST expression in rat liver were obtained using cytosolic extracts whereas those in the cultured cells were obtained using total cell homogenates. Hence, unlike liver cytosolic extracts, total cell homogenates will not be enriched for GST proteins and, in comparison with rat liver, will therefore appear to express very low amounts of GST proteins.

When results were normalised in the way described above, we observed that the average amount of GST1-1/2-2 proteins expressed in untreated primary hepatocyte cultures was approximately 8.9 % of the amount expressed in the liver, whereas the amount of GST3-3/4-4 proteins was approximately 7 %. Hence, the percentage of GST1-1/2-2 and 3-3/4-4 expression in cultured cells, compared with the expression of the same proteins in liver, remained constant. This result suggests that the culture conditions under investigation did not cause any major alteration in the expression of GST1-1/2-2 in relation to GST3-3/4-4. Hence, the decrease in GST1-1/-2-2 and the increase in GST3-3/4-4, observed in

previous culture systems (Vandenberghe et al., 1988, 1989, 1990) and associated with foetal hepatocytes, did not appear to occur. Perhaps the most significant indication that the culture conditions support expression of GST proteins in a way that resembles the situation in the liver, is the fact that no GST7-7 expression could be detected in these cells, neither in untreated nor in phenobarbital treated cells. In this respect, this cell system compares very favourably with cell systems developed during the course of other investigations (Abramovitz et al., 1989; Vandenberghe et al. 1988, 1990). Finally, in contrast to phase I drug-metabolising enzymes, Vitrogen coating of culture plates did not cause any significant improvement in the expression of GST proteins.

3.4 Immortalisation of primary hepatocytes

The primary hepatocyte system developed in this study reflects the liver with regards to expression and induction of members of the CYP2B and GST family. However, because of their relatively short life in culture, it is necessary to isolate fresh hepatocytes each time a new experiment is to be performed. This makes hepatocyte culture systems costly, not only in economical terms, but also from an ethical point of view since it is necessary to sacrifice an animal for each experiment. Established cell lines have the obvious advantage of being readily available. For the study of drug metabolising enzymes we have demonstrated that it is possible to utilise highly differentiated cell lines, such as FAZA 967 cells, to reproduce at least in part phenotypes normally observed in the intact liver. However, these cells are fully-transformed and are obviously very different from hepatocytes present in the liver. A good compromise would therefore be a cell system constituted by immortalised hepatocytes, in which cells, although minimally transformed, were able to divide under certain conditions. A subgroup of cellular and viral oncogenes has the ability to establish continuous proliferation in the culture of primary cells. The viral oncogenes which display immortalising activity are adenovirus E1a, simian virus 40 (SV40) and polyoma virus large T-antigen genes (Rassoulzadegan et al., 1982; Petit et al., 1983; Jat & Sharp, 1986; Phelps et al., 1988). The cellular oncogenes with immortalising activity are *myc, fos* and p53 (Eliyahu et al., 1984; Jenkins et al., 1984). In addition to its immortalising activity, expression of the SV40 large T-antigen has been shown to lead to at least some stabilisation of the cell-type specific functions (Zaret et al., 1988; Paul et al., 1988; Nemunaitis et al., 1989; De Vitry et al., 1974; Moura Neto et al., 1986). For this reason, in an attempt to establish an immortalised hepatocyte cell system, we isolated hepatocytes from a mouse harbouring a temperature-sensitive mutant of the SV40 large T antigen gene under the control of the mouse major histocompatibility complex H-2K^b class II promoter which is inducible by γ -interferon (Jat et al., 1991; Fig. 3.27). The trangenic mice used in these experiments

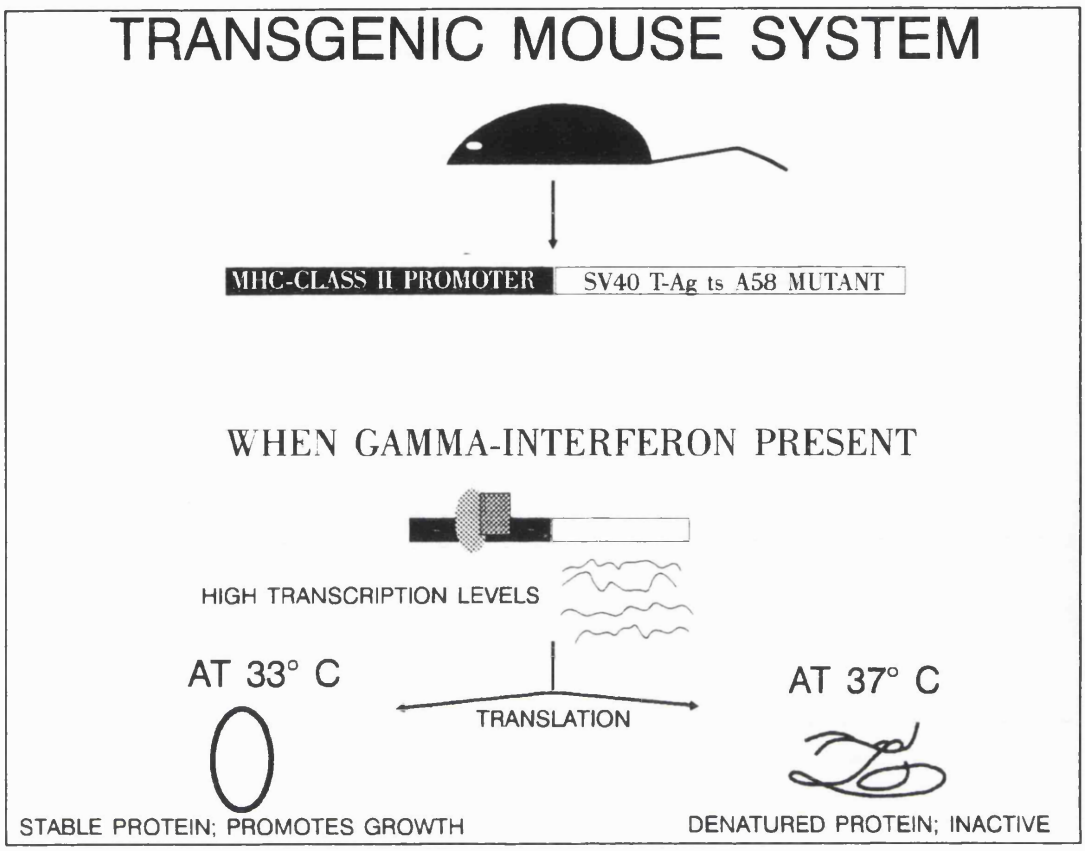


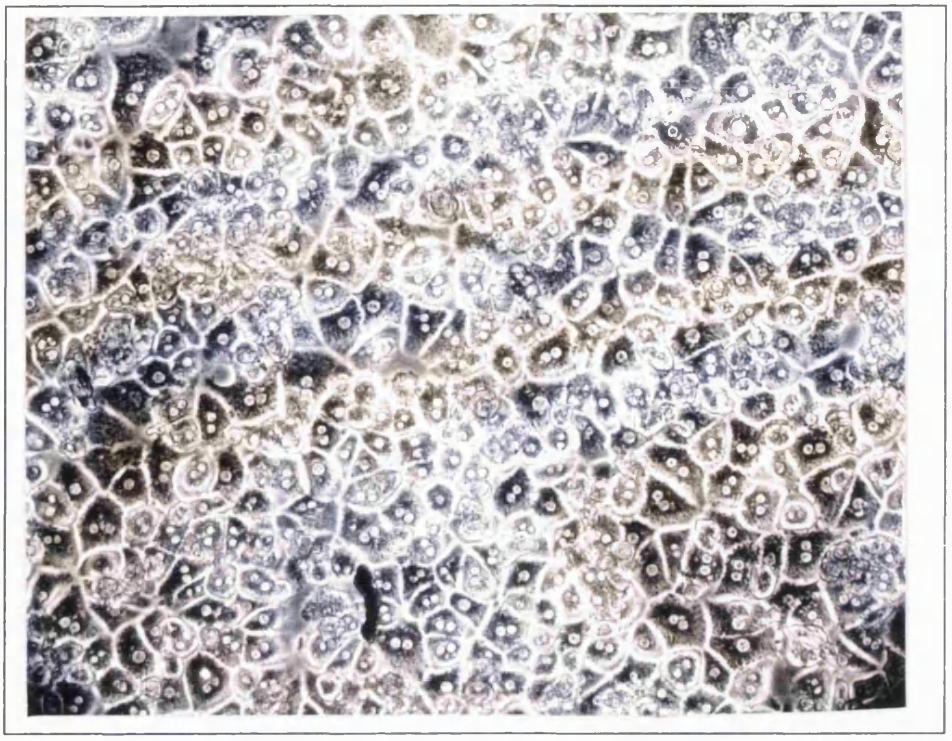
Fig. 3.27 Schematic representation of the transgenic mouse system

were a kind gift from Prof. Mark Noble, The Ludvig Institute, London, U. K. The permissive temperature for the gene product is 33° C; at the non-permissive temperature of 37° C it is rapidly degraded. Theoretically, cells isolated from these transgenic mice should be able to divide and be in a dedifferentiated state at the permissive temperature and should be able to stop dividing and differentiate at the non-permissive temperature.

Mouse hepatocytes were isolated with good success using the protocol utilised for the isolation of rat hepatocytes, although viability levels were slightly lower than those achieved with rat hepatocytes (>75 %). Livers for these experiments were isolated from 8 week-old mice, heterozygotes for the transgenic gene. In order to allow expression of active large T antigen at the earliest possible time, isolation was carried out at 35° C, rather than at 37 ° C, without any major alteration in cell viability or yield.

As was the case for rat hepatocytes, we initially compared various culture conditions in order to find one that supported good cell attachment, survival and morphology. William's E medium and Chee's Modified Eagles' medium (CMEM) were tried in combination with Vitrogen-coated or uncoated Permanox, Primaria or Nunc plates. Both media were supplemented with insulin, dexamethasone, transferrin and hydrocortisone since from the literature (Clayton & Darnell, 1983; Paul et al., 1988; Yanai et al., 1991) these conditions appeared to be particularly well suited for the culture of mouse hepatocytes. Best results were obtained when cells were plated on Vitrogen-coated Permanox plates in the presence of William's E medium. Under these conditions, if cells were plated at high cell density (5 x 10^6 cells/60 mm plate) they formed a monolayer whose appearance closely resembled that of a normal primary hepatocyte culture (Fig. 3.28). After passaging, cells tended to grow in patches rather than as single cells (Fig. 3.29, panels A and B). Confluence was re-established after 7-10 days (Fig. 3.29, panel C). In order for cell division to take place, EGF was found to be indispensable. The hepatocytes obtained after passaging are mainly mono- or binucleated but some polynucleated cells could be observed.

A



B

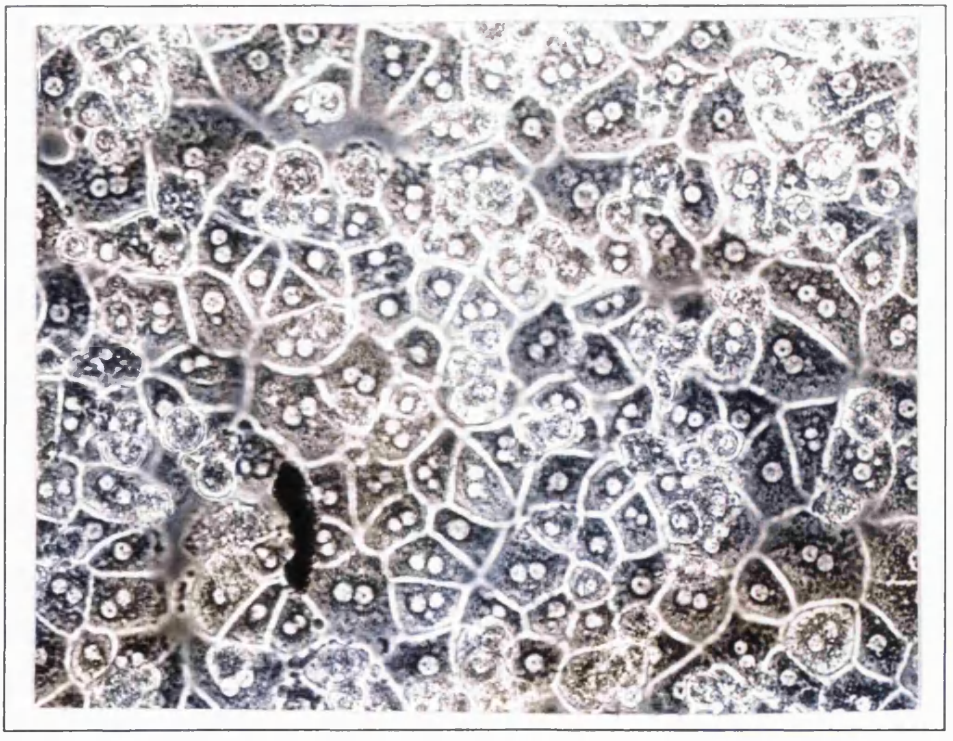


Fig. 3.28 Light photomicrographs of transgenic mouse hepatocytes cultured at high density (5 x 10^6 cells/60 mm plate) on Vitrogen-coated Permanox plates. (**A**) x 40 magnification, (**B**) x 80 magnification.

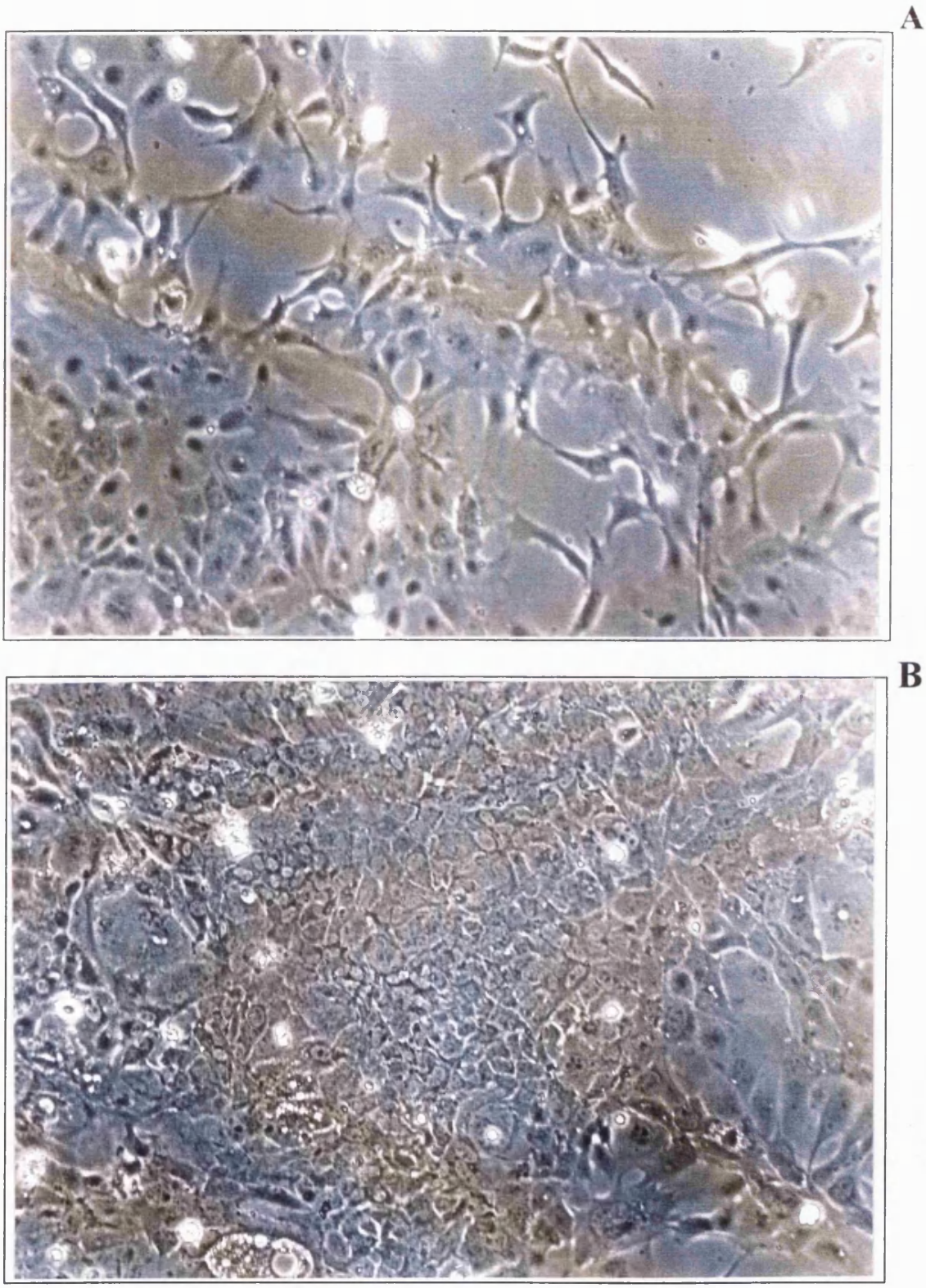


Fig. 3.29 Light photomicrographs of transgenic mouse hepatocytes after passaging (continued on next page). (**A**) Immediately after passaging cells start to aggregate into patches. (**B**) Islands of cells are clearly visible 2-3 days later. In the centre of the islands, tight connections among neighbouring cells are re-established.

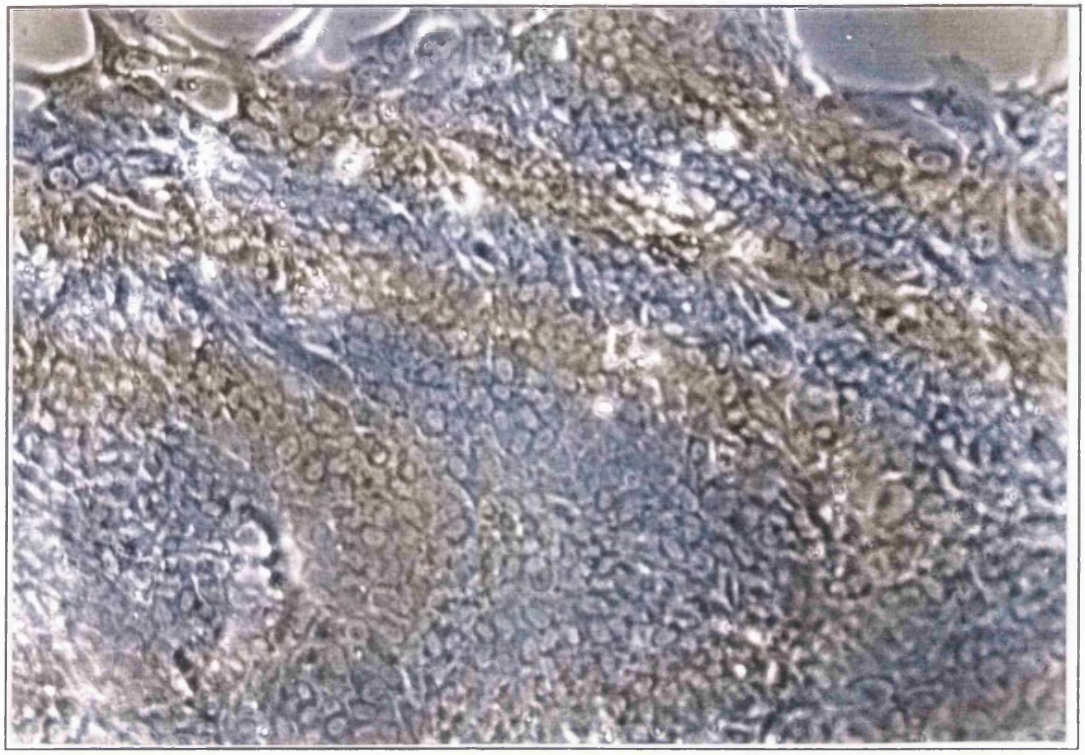


Fig. 3.29 Light photomicrograph of transgenic mouse hepatocytes after passaging (continued from previous page). (**C**) Confluence is re-established 7-10 days after passaging (plate ∅ = 60 mm).

The cells have been maintained in culture for several generations by continuous passaging and during this time they retained, both at 33° C and 37° C, the characteristic morphology associated with differentiated adult hepatocytes. To begin to assess the extent to which these cells express liver-specific functions, secretion of serum albumin was monitored using a clinical analyser at Glaxo Group Research Ltd, Ware, UK. An aliquot of fresh medium, containing all supplements, was used as the reference sample. In order to determine that the albumin detected in the culture medium was due to secretion and not to a general leakiness of the cells, the presence of Lactate Dehydrogenase (LDH) in the medium was also monitored. The results, shown in Fig. 3.30, indicate that serum albumin was secreted at constant levels throughout the thirty days in culture. The very low levels of LDH detected in the medium were consistent with values expected from healthy, non-leaky hepatocyte cultures (Jauregui et al., 1981), suggesting that the presence of albumin in the medium was due to secretion and not to leakiness. The large peak in serum albumin secretion observed at 21 days in culture was associated with an increase in LDH levels. However, the standard deviation from the mean value was also large at this point, suggesting that only some of the samples tested at this time-point were collected from cultures that were damaged. Consequently, the values determined at this time-point should not be considered as representative of the general trend .

Further characterisation of these cells is currently being carried out in the laboratory of Dr. Ian Phillips at Queen Mary and Westfield College, London, UK. Here it has been shown by RNase protection that these cells express CYP1A1 mRNA when cultured in the presence of β-naphtoflavone (Kramer et al., 1994). Probes have also been constructed for RNase protection assay of mRNAs encoding CYPs 2B, 1A2, 3A and cytochrome b5. The expression and inducibility of these mRNAs in the immortalised hepatocytes is currently being investigated.

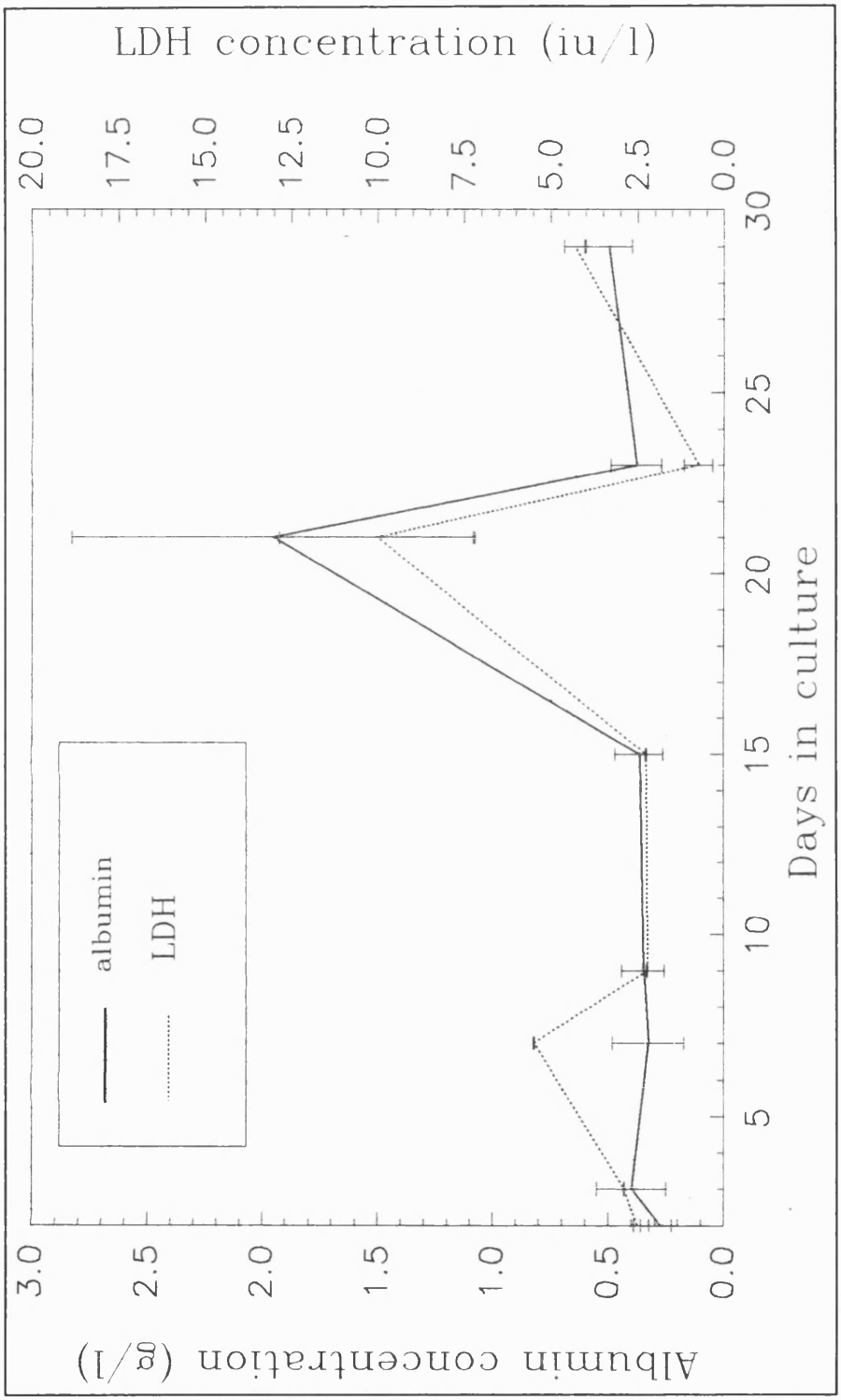


Fig. 3.30 Albumin versus LDH secretion in transgenic mouse hepatocytes. Points represent mean values/10^6 cells (±SD; n>5). The concentration of both proteins was detected by means of a clinical analyser in aliquots of media in which cells had been cultured for 24 hr.

3.5 Initial attempts to use the primary hepatocytes system to map regions of the *CYP2B2* gene promoter important for regulation.

Having satisfied the initial requirements of the project, we wanted to investigate whether it was possible to use the cell systems developed in the course of this study in transfection experiments (reviewed in Alam & Cook, 1990) aimed at defining regions of the *CYP2B2* gene important for its regulation. Because best induction responses to PB were observed in primary hepatocytes, we decided to concentrate on this cell system. Successful protocols for the transfection of primary hepatocytes have been reported in the literature (Muakkassah-Kelly et al., 1988; Ginot et al., 1989; Rippe et al., 1990; Parker-Ponder et al., 1991; Jarnagin et al., 1992; Burger et al., 1992). However, in examining the various protocols we needed to bear in mind the phenomenon of the culture-related changes that affect the ability of these cells to maintain an induction response to phenobarbital. Particular attention had to be paid to the overall timing of the transfection procedure, bearing in mind that best induction responses appeared to be elicited 48 hr after addition of the inducer. Moreover, all transfection procedures have to be performed on cells that were plated on Vitrogen-coated Permanox plates, since this plate-substratum combination in our cell system appeared to stabilise the hepatocyte phenotype. For these reasons we tested several modifications of various transfection protocols in order to find conditions that efficiently allowed DNA transfer into primary hepatocytes cultured under conditions developed in the course of this study. In order to compare transfection efficiencies, cells were transfected with the plasmid pCMVβ-gal (purchased from Clontech, UK). This is a reporter gene vector, in which the major immediate early promoter of human cytomegalovirus (CMV) has been cloned immediately upstream of the *E. coli* β-galactosidase gene. The CMV promoter has been shown to be particularly active in primary rat hepatocytes (Jarnagin et al., 1992). Expression of the β-galactosidase enzyme was monitored by histochemical staining with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Cells actively expressing β-

galactosidase were readily identified by their dark blue colour (Fig. 3.31). A summary of the results is given in Table 3.1. Despite trying several combinations of the protocol described in Muakkassah-Kelly et al. (1988) in our hands electroporation caused extensive cell death. Calcium-phosphate precipitation protocols also proved unsuccessful (Ginot et al., 1989; Rippe et al., 1990). However, an enhancement of the calcium phosphate technique, in which uptake of the $CaPO_4$/DNA complex is forced by a "*glycerol shock*" (Rosenthal, 1987) was successful, although transfection efficiency was still quite low (≈ 1-2 %). In contrast, we could not repeat the success of another group (Chen and Okayama, 1987) in which the "*glycerol shock*" is substituted by a "*BES shock*", using a substance known as **BES** [*N, N*-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid]. A modification of the lipofection protocol described in Burger et al. (1992) was instead highly successful, with transfection efficiencies ranging between 10 and 20 %. An example of cells transfected in this way and stained *in situ* with X-gal is shown in Fig. 3.31.

Table 3.1 Comparison of transfection efficiencies achieved by different techniques in primary hepatocytes and FAZA 967 cells

	Primary hepatocytes	FAZA 967
Electroporation	0	0
Calcium phosphate	0	0
Calcium phosphate ("glycerol shock")	1-2	0
Calcium phosphate ("BES shock")	0	0
Lipofection	10-20	1-2

Transfection efficiency was measured as the percentage of cells stained blue after incubation with X-gal.

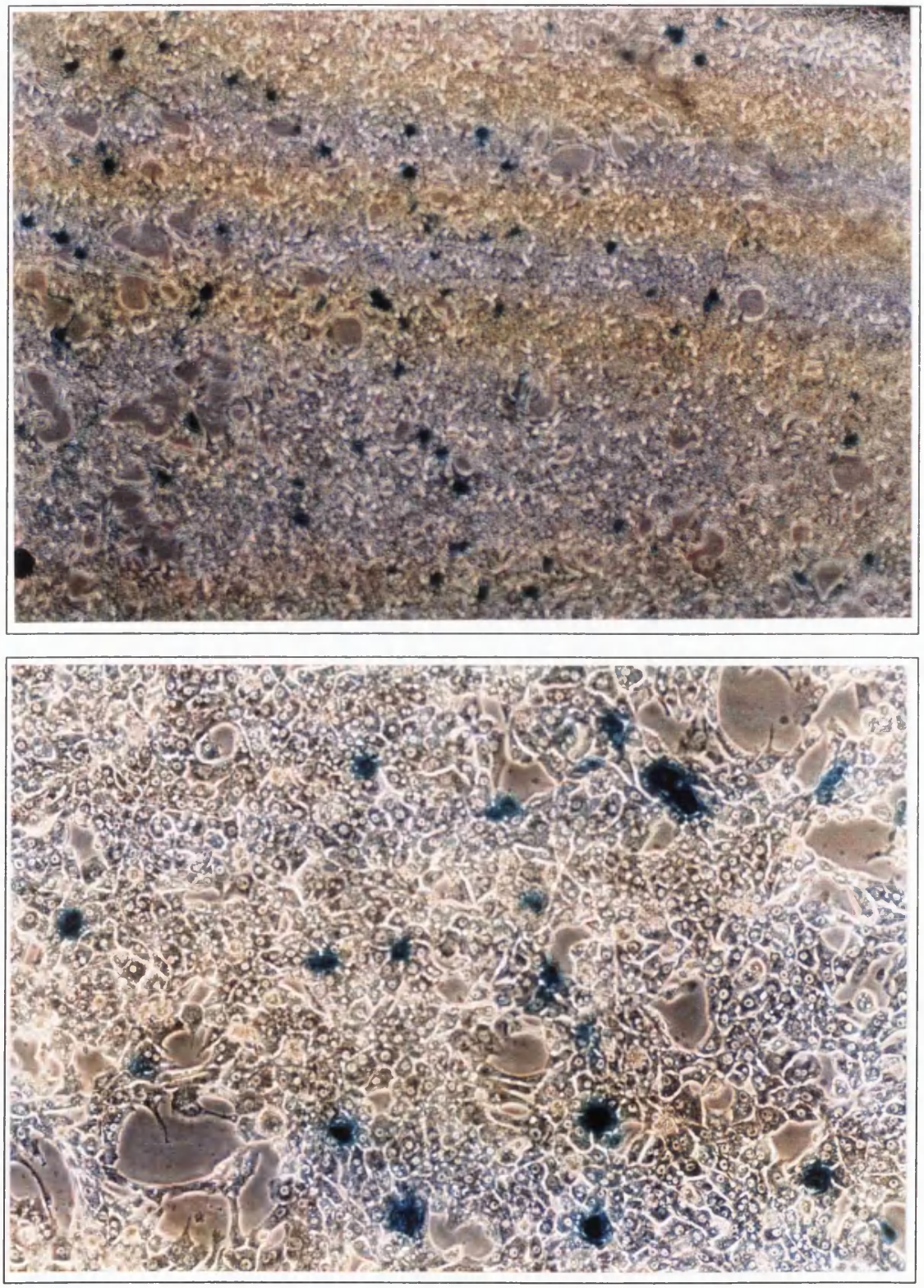


Fig. 3.31 Light photomicrographs of primary hepatocytes transfected by lipofection with pCMVβ-gal, after *in situ* staining with X-gal. (**A**) x 10 magnification; (**B**) x 40 magnification

The same approach taken to optimise conditions for efficient transfection of DNA into primary hepatocytes was taken also for FAZA 967 cells. Contrary to our expectations these cells proved harder to transfect. In this case also, best results were obtained with lipofection, however transfection efficiency was never higher than 1-2 % (Table 3.1).

Having set up a protocol for the efficient transfer of DNA into primary hepatocytes, we attempted to use this system to study the *CYP2B2* gene promoter. On the basis of evidence accumulated in the course of previous *in vitro* studies performed in our laboratory (Shephard et al., 1994; Shervington et al., 1994; Shervington et al., unpublished observations), we decided to concentrate on the 5'-flanking region of a *CYP2B2* gene (Shephard et al., 1994) that is comprised between nucleotides -368 and -4. This region has been shown to contain DNA elements that, in gel-retardation and in *vitro* footprinting assays (Shephard et al., 1994), can interact with proteins that are either more abundant or more active in nuclei isolated from the liver of phenobarbital-treated rats, than proteins in nuclei isolated from untreated rats. Moreover, *in vitro* transcription assays have shown that when this region of the *CYP2B2* gene interacts with extracts isolated from the liver of phenobarbital treated rats, it is able to catalyse the accumulation of heterologous transcripts at levels 2- to 3-fold higher than when it is allowed to interact with extracts isolated from untreated rats (Shervington et al., 1994; Shervington et al., in preparation).

The reporter gene vector we decided to use in our experiments is the pGL2-enhancer plasmid (Promega, UK). The reporter gene cloned in this plasmid encodes firefly (*Photynus pyralis)* luciferase. This enzyme catalyses the following reaction (Shaw, 1975):

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{Mg^{++}} \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

Under appropriate reaction conditions, both the intensity of the flash and the integrated light output values are, over a period of time, directly proportional to the

amount of enzyme present in the reaction (deWet et al., 1985). Hence, it is possible to measure the amount of reporter gene expression as a function of light emitted using either a luminometer (light intensity) or a scintillation counter (integrated output value; Nguyen et al., 1988). Because mammalian cells do not possess an activity similar to that of luciferase, the sensitivity of this reporter gene system is very high [typically 2 to 3 orders of magnitude higher than systems using chloramphenicol acetyl transferase (CAT) as the reporter gene (Alam & Cook, 1990)]. Moreover, luciferase assays are less expensive and time-consuming than CAT assays. Therefore, firefly luciferase appears to be the reporter gene of choice for analysing the transcriptional activity of weak promoters and examining promoter function in cells that do not readily take up DNA, and hence ideally suited for the experiments we intended to perform.

In order to determine the ability of the -368/-4 region of *CYP2B2* to direct the expression of a reporter gene, we subcloned the -368/-4 region immediately upstream of the luciferase gene, to generate the plasmid p(-368/-4)*luc* and transfected this construct into primary cells. To ensure that any results obtained with p(-368/-4)*luc* could be normalised against transfection efficiency, this plasmid was cotransfected with pCMVβ-gal. In order to determine whether transcription directed the -368/-4 region could be augmented by phenobarbital, after transfection was completed, the media of some of the cultures were supplemented with the barbiturate. As a positive control in our experiments, we also transfected some of the cultures with the plasmid pCMV-luc (prepared by Dr. Shervington). This is a derivative of the pGL2-enhancer plasmid, in which the CMV major early promoter was subcloned immediately upstream of the luciferase gene. The strong activity of the CMV promoter in these cells, should ensure transcription of the luciferase gene. If it is possible to generate active luciferase in our cell system, we should certainly be able to observe a positive signal in cells transfected with this plasmid.

Despite the fact that light emission could be observed in total cellular extracts

isolated from cells transfected with pCMV-luc (Fig. 3.32), we could detect luciferase activity neither in untreated, nor in phenobarbital-treated cells. Several factors may have contributed to this negative result. A likely cause might have been the fact that during the transfection procedure cells were subject to damage, causing phenotypic changes that do not allow the cell system to be responsive to phenobarbital. However, CYP2B1/2 mRNA levels were increased in cultures that, in a parallel experiments, had been "mockly" transfected and subsequently cultured in the presence of PB (data not shown). It is possible therefore, that even if the cells are still responsive to PB, the -368/-4 region of the *CYP2B2* promoter is not sufficient to reproduce a phenobarbital response and that larger or different 5'-flanking regions of the *CYP2B2* promoter may be required. Moreover, it is also possible that in our cell system luciferase gene transcripts are translated inefficiently, possibly due to inefficient modification of their 5'-end (translation mechanisms and their regulation are reviewed in Hershey, 1991; Rhoads, 1991; Merrick, 1992). The fact that the signal observed in cultures transfected with pCMV-luc is not particularly strong (Fig. 3.32) may be an indication that this is the case. With a strong promoter such as the CMV major immediate early promoter, because of the large number of transcripts generated, translation efficiency becomes of secondary importance, and a signal, albeit weak, can still be observed. However, with a weak promoter such as the -368/-4 region, translation becomes a rate limiting step. If translation is not efficient, it is possible that luciferase gene transcripts are degraded more rapidly than they are generated, accumulating luciferase enzyme at levels below the limit of detection of the assay employed. More efficient translation of luciferase gene transcripts may be accomplished by engineering the reporter gene cassette so that its transcription generates an mRNA molecule with the same 5' non-translated region of CYP2B2 transcripts. Alternatively, it should be possible to engineer the reporter gene cassette so that the cDNA for an internal ribosomal entry site (IRES; reviewed in McBratney et al., 1993) is positioned immediately upstream of the luciferase gene.

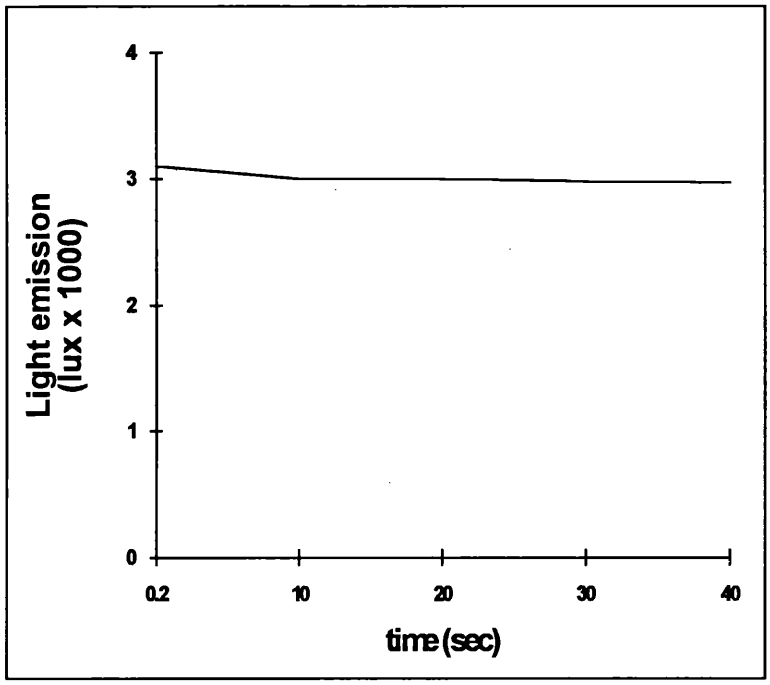


Fig. 3.32 Result of a luciferase assay performed on total cell extracts isolated from primary rat hepatocytes transfected with pCMV-luc.

It is evident from these initial experiments that much work is still needed in order to map regions of the *CYP2B2* promoter involved in regulation. Time constraints on this project have not allowed me to continue further in this investigation. However, we have demonstrated that it is possible to transfect DNA into primary hepatocytes with high efficiency, under culture conditions that allow these cells to express an adult liver phenotype. This cell system should therefore be ideally suited to study the promoters of *CYP2B* genes.

Chapter four

Conclusions

During the course of this study we have set up culture conditions that permit primary rat hepatocytes to express several genes encoding phase I and phase II drug-metabolising enzymes, namely CYP2B1/2, P450 reductase, FMO1, and GSTs 1-1/2-2, and 3-3/4-4. Levels of expression of these genes closely resembled those observed in rat liver. Moreover, as is the case in liver cytosolic extracts, GST7-7 expression was undetectable in these cells. Similar to the situation *in vivo,* in primary hepatocytes cultured under these conditions, phenobarbital was shown to strongly stimulate the accumulation of CYP2B1/2 mRNAs and proteins. To a lesser extent, PB was also shown to promote the accumulation of P450 reductase, GST1-1/2-2, and GST3-3/4-4 proteins. During the course of this study we have also shown that in this culture system picrotoxin strongly induces CYP2B1/2 mRNAs, with a kinetics that closely resembles that observed using phenobarbital as the inducer. Picrotoxin was found to induce also the expression of other drug-metabolising enzymes according to a pattern that closely resembles that observed using phenobarbital. These observations strongly suggest that picrotoxin and phenobarbital might induce the expression of drug-metabolising enzymes, in general, and of CYP2B1/2 mRNAs, in particular, via a similar mechanism, possibly mediated by an intracellular receptor protein. Altogether these data are a strong indication of the overall ability of this cell system to constitute a valid *in vitro* model for the study of drug metabolism, at least on a short term basis. The chemically-defined culture conditions that we employed are relatively simple, free from complicating factors such as Matrigel or co-culture (discussed in section 1.3). In addition, we have shown that cells cultured under these conditions can be transfected with high efficiency and that luciferase reporter gene constructs are active in these cells. Therefore, this cell system can be readily employed not only for metabolic studies, but also for studies aimed at understanding the regulation of the expression of genes encoding drug-metabolising enzymes. The major problem associated with the primary hepatocyte cell system is the fact that cells need to be isolated each time a new experiment is started. In our efforts to overcome this problem,

we have shown that the culture conditions developed for primary hepatocytes can be employed for the culture of FAZA 967 rat hepatoma cell line. These conditions were found to support the expression of several drug-metabolising enzymes in a manner that was similar to that observed in primary hepatocytes. Although in these cells the fold-induction of CYP2B1/2 mRNAs by phenobarbital and picrotoxin was comparable to the fold induction observed in primary hepatocytes, expression levels for the two mRNAs was considerably lower. It is doubtful, therefore, whether this cell system can be used for the study of the metabolism of drugs by CYP2B1/2. Nonetheless, the culture conditions developed in the course of this study were found to support the expression of GST1-1/2-2, FMO1, and P450 reductase in a manner that is comparable to that observed in primary hepatocytes. In addition, these cells expressed GST7-7, and the expression of P450 reductase and GST1-1/2-2, and GST7-7 was found to be inducible by phenobarbital. Hence, considering the great advantages of an established cell line over a primary hepatocyte system, the FAZA 967 system developed in the course of this study should represent an ideal *in vitro* model for the study of the drug-metabolising enzymes mentioned above.

Finally, we have established conditions that allow primary mouse hepatocytes, isolated from a transgenic mouse harbouring a temperature-sensitive mutant of the SV40 large T antigen, to divide and survive in culture for longer than a month. These cells could be passaged and were found to secrete albumin. Kerstin Kramer has continued this work in Dr. Ian Phillips' laboratory at Queen Mary and Westfield College, London, and found that these cells could express high levels of CYP1A2 mRNA when cultured in the presence of β-naphtoflavone. She has now passaged the cells over 30 times and has cloned nine cell lines. Characterisation of these clones is now in progress.

In conclusion, the three cell systems developed in the course of this project have the potential to contribute collectively to a substantially better understanding of drug-metabolism. Considering that an *in vitro* system can never represent more than an

approximation of what occurs in the liver, it is reasonable to use a system that is simple to assemble, to use, and whose results can be readily interpreted. The primary hepatocyte system developed in this study offers this advantage over cell systems developed in the past. In addition, the expression of the phase I and phase II enzymes investigated compares very favourably with previously developed cell systems, at least during four days in culture. Longer culture periods were not investigated and it could be possible that the culture conditions developed in this project might allow cells to survive in culture for longer. In this case this system could also be used for long-term drug-metabolism studies. It would be interesting also to investigate drug-metabolism as a function of cell division in the immortalised cell lines developed. It is possible to conditionally regulate whether these cells divide or not in culture. Therefore, it should be possible to mimic in part what occurs in the liver during regeneration, following damage. In fact, the mechanisms involved in the regulation of liver regeneration are largely unknown and this cell system could be employed in studies aimed at elucidating these mechanisms.

Bibliography

Abramovitz, M., Ishigaki, S. and Listowsky, I. (1989). *Hepatology* **9**: 235-239.

Abramovitz, M. and Listowsky, I. (1987). *J. Biol. Chem.* **262**: 7770-7775.

Abramovitz, M. and Listowsky, I. (1988). *Xenobiotica* **18**: 1249-1254.

Agosin, M. and Ankley, G. T. (1987). *Drug. Metab. Disp.* **15**: 200-203.

Akrawi, M., Rogiers, V., Vandenberghe, Y., Palmer, C. N., Vercruysse, A., Shephard, E. A. and Phillips, I. R. (1993). *Biochem. Pharmacol.* **45**: 1583-1591.

Al Waiz, M., Ayesh, R., Mitchell, S. C., Idle, J. R. and Smith, R. L. (1987). *Lancet* **i**: 634-635.

Al Waiz, M., Mitchell, S. C., Idle, J. R. and Smith, R. L. (1987b). *Xenobiotica* **17**: 551-558.

Alam, F. and Cook, J. L. (1990). *Anal. Biochem.* **188**: 245-254.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987). *Cell* **49**: 729-739.

Bains, S. K., Gardiner, S. M., Mannweiler, K., Gillett, D. and Gibson, G. G. (1985). *Biochem. Pharmacol.* **34**: 3221-3229.

Ball, S. and Bruice, T. (1986). *J. Biol. Chem.* **261**: 2553-2559.

Baumann, H. (1989). *In Vitro Cell Dev. Biol.* **25**: 115-126.

Bayad, J., Sabolovic, N., Bagrei, D., Magdalou, J. and Siest, G. (1991). *J. Pharmacol. Meth.* **25**: 85-94.

Bellward, G. D., Chang, T., Rodrigues, B., McNeill, J. H., Maines, S., Ryan, D., Levin, W. and Thomas, P. E. (1988). *Mol. Pharmacol.* **33**: 140-143.

Ben-Ze'ev, A., Robinson, G. S., Buchner, N. L. and Farmer, S. R. (1988). *Proc. Natl. Acad. Sci.* **85**: 2161-2165.

Berg, T., Boman, D. and Seglen, P. O. (1972). *Exp. Cell Res.* **72**: 571-574.

Berry, M. N. and Friend, D. S. (1969). *J. Cell Biol.* **43**: 506-520.

Black, S. and Coon, M. (1986). In: *Cytochrome P-450: Structure, Mechanism and Biochemistry.* (P. R. Ortiz de Montellano, Ed.), pp.161-216, Plenum, New York.

Black, S. D. (1992). *FASEB J.* **6**: 680-685.

Board, P. G. (1981). *Am. J. Genet.* **33**: 36-43.

Boyer, T. D. (1989). *Hepatology* **9**: 486-496.

Brodie, M. J. (1990). *The Lancet* **336**: 350-354.

Buhler, R., Lindros, K. O., Nordling, A., Johansson, I. and Ingelman-Sundberg, M. (1992). *Eur. J. Biochem.* **204**: 407-412.

Burbach, B., Poland, A. and Bradfield, C. A. (1992). *Proc. Natl. Acad. Sci.* **89**: 8185-8189.

Burchell, B., Nebert, D. W., Nelson, D. R., Bock, K. W., Iyanagi, T., Jansen, P. L., Lancet, D., Mulder, G. J., Chowdhury, J. R., Siest, G. and et, a. l. (1991). *DNA Cell Biol.* **10**: 487-494.

Burger, H.-J., Schuetz, J. D., Shuetz, E. G. and Guzelian, P. S. (1992). *Proc. Natl. Acad. Sci.* **89**: 2145-2149.

Burke, M. D., Thompsom, S., Elcombe, C. R., Halpert, J., Haaparanta, T. and Mayer, R. T. (1985). *Biochem. Pharmacol.* **34**: 3337-3345.

Campbell, E., Takahashi, Y., Abramovitz, M., Perez, M. and Listowsky, I. (1990). *J. Biol. Chem.* **265**: 9188-9193.

Carpenter, G. and Wahl, M. I. (1990). In: *Peptide growth factors and their receptors.* (M. D. Sporn and A. B. Roberts, Ed.), pp.69-171, Springer-Verlag, New York.

Casazza, J. P., Felver, M. E. and Veech, R. L. (1984). *J. Biol. Chem.* **259**: 231-236.

Chen, C. and Okayama, H. (1987). *Mol. Cell. Biol.* 7: 2745-2752.

Chianale, J., Dvorak, C., May, M. and Gumucio, J. J. (1986). *Hepatology* **6**: 945-951.

Cholerton, S., Daly, A. K. and Idle, J. R. (1992). *TIPS* **13**: 434-439.

Christou, M., Wilson, N. M. and Jefcoate, C. R. (1987). *Arch. Biochem. Biophys.* **258**: 519-534.

Ciliberto, G. (1989). In: *Acute Phase Proteins and the Acute Phase Response*. (M. Pepys, Ed.), pp.29-46, Springer-Verlag, New York.

Ciliberto, G., Colantuoni, V., De Francesco, R., De Simone, V., Monaci, P., Nicosia, A., Ramji, D. P., Toniatti, C. and Cortese, R. (1993). In: *Gene Expression: General and Cell-Type Specific*. (M. Karin, Ed.), pp.162-242, Birkhauser, Boston.

Clayton, D. F. and Darnell, J. E. (1983). *Mol. Cell. Biol.* **3**: 1552-1561.

Coecke, S., Segaert, A., Vercruysse, A. and Rogiers, V. (1993). *Toxicology in vitro* **7**: 487-491.

Connelly, J. C. and Bridges, J. W. (1980). *Prog. in Drug Metab.* **5**: 1-109.

Conney, A. H. (1967). *Pharmacol. Rev.* **19**: 317-366.

Conney, A. H. (1982). *Cancer Res.* **42**: 4875-4917.

Cowell, I. G., Dixon, K. H., Pemble, S. E., Ketterer, B. and Taylor, J. B. (1988). *Biochem. J.* **255**: 79-83.

Dalet, C., Clair, P., Daujat, M., Fort, P., Blanchard, J.-M. and Maurel, P. (1988). *DNA* 7: 39-46.

Daniel, V. (1993). *CRC Crit. Rev. Biochem. Mol. Biol.* **28**: 173-207.

Daniel, V., Sharon, R. and Bensimon, A. (1989). *DNA* **8**: 399-408.

Daniel, V., Sharon, R., Tichauer, Y. and Sarid, S. (1987). *DNA* **6**: 317-323.

Dannan, G. A. and Guengerich, F. P. (1982). *Mol. Pharmacol.* **22**: 787-794.

Dannan, G. A., Guengerich, F. P., Kaminsky, L. S. and Aust, S. D. (1983). *J. Biol. Chem.* **258**: 1282-1288.

De Jong, J. L., Chang, C. M., Whang-Peng, J., Knutsen, T. and Tu, C.-P. D. (1988b). *Nucleic Acids Res.* **16**: 8541-8546.

De Jong, J. L., Morgenstern, R., Jornvall, H., De Pierre, J. W. and Tu, C.-P., D. (1988). *J. Biol. Chem.* **263**: 8430-8435.

De Long, M. I., Santamaria, A. B. and Talalay, P. (1987). *Carcinogenesis* **8**: 1549.

De Simone, V. and Cortese, R. (1991). *Curr. Opin. Cell Biol.* **3**: 960-965.

De Vitry, F., Camier, M., Czernichow, P., Benda, P. H., Choen, P. and Tixier-Vidal, A. (1974). *Proc. Natl. Acad. Sci.* **71**: 3575-3579.

de Wet, J. R., Wood, K. V., Helinski, D. R. and DeLuca, M. (1985). *Proc. Natl. Acad. Sci.* **82**: 7870-7873.

Denison, M. S., Vella, L. M. and Okey, A. B. (1986). *J. Biol. Chem.* **261**: 3987-3995.

Deschatrette, J. and Weiss, M. C. (1974). *Biochimie* **56**: 1603-1611.

Ding, G. J.-F., Ding, V. D.-H., Rodkey, J. A., Bennet, C. D., Lu, A. Y. H. and Pickett, C. B. (1986). *J. Biol. Chem.* **261**: 7952-7956.

Ding, G. J.-F., Lu, A. Y. H. and Pickett, C. B. (1985). *J. Biol. Chem.* **260**: 13268-13272.

Dixon, K. H., Cowell, I. G., Xia, C. L., Pemble, S. E., Ketterer, B. and Taylor, J. B. (1989). *Biochem. Biophys. Res. Comm.* **163**: 815-819.

Dolphin, C., Shephard, E. A., Povey, S., Palmer, C. A., Ziegler, D. M., Ayesh, R., Smith, R. L. and Phillips, I. R. (1991). *J. Biol. Chem.* **266**: 12379-12385.

Dolphin, C. T., Shephard, E. A., Povey, S., Smith, R. L. and Phillips, I. R. (1992). *Biochem J.* **287**: 261-267.

Dunn, T. J., Koleske, A. J., Lindahl, R. and Pitot, H. C. (1989). *J. Biol. Chem.* **264**: 13057-13065.

Eichelbaum, M. and Gross, A. S. (1990). *Pharmacol. Ther.* **46**: 377-394.

Eliyahu, D., Raz, A., Gruss, P., Girol, D. and Open, M. (1984). *Nature* **312**: 646-649.

Elshourbagy, N. A. and Guzelian, P. S. (1980). *J. Biol. Chem.* **255**: 1279-1285.

Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N., Gotoh, O., Funae, Y. and Fujii-Kuriyama, Y. (1992). *Biochem. Biophys. Res. Commun.* **184**: 246-253.

Emi, Y., Chijiiwa, C. and Omura, T. (1990). *Proc. Natl. Acad. Sci.* **87**: 9746-9750.

Endicott, J. A. and Ling, V. (1989). *Annu. Rev. Biochem.* **58**: 137-171.

Estabrook, R. W. (1978). In: *Methods in Enzymology*. vol. LII, (S. Fleischner and L. Packer, Ed.), pp.43-47, Academic Press, New York.

Falany, C. N. (1991). *Trends Pharmacol. Sci.* **12**: 255-259.

Fey, G. H. and Gauldie, J. (1990). In: *Progress in Liver Diseases*. vol. 9, (H. Popper and F. Shaffner, Ed.), pp.89-116, WB Saunders, Philadelphia.

Fourney, R. M., Miyakowski, J., Day, R. S. and Paterson, M. C. (1988). *BRL Focus* **10**: 5-6.

Fowler, A. V. and Zabin, I. (1983). *J. Biol. Chem.* **258**: 14354-14358.

Friling, R. S., Bensimon, A., Tichauer, Y. and Daniel, V. (1990). *Proc. Natl. Acad. Sci.* **87**: 6258-6262.

Fujii-Kuriyama, Y., Imataka, H., Sogawa, K., Yasumato, K. and Kikuchi, Y. (1992). *FASEB J.* **6**: 706-710.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K. and Murmatsu, M. (1982). *Proc. Natl. Acad. Sci.* **79**: 2793-2797.

Fysh, J. M. and Okey, A. B. (1978). *Biochem. Pharmacol.* **27**: 2968-2972.

Garrison, J. C. and Haynes, R. C. (1973). *J. Biol. Chem.* **248**: 5333-5343.

Gasser, R., Tynes, R. E., Lawton, M. P., Korsmeyer, K. K., Ziegler, D. M. and Philpot, R. M. (1990). *Biochemistry* **29**: 119-124.

Gebhardt, R. (1992). *Pharmacol. Ther.* **53**: 275-354.

Giachelli, C. M. and Omiecinski, C. J. (1986). *J. Biol. Chem.* **261**: 1359-1363.

Gibson, G. G. (1992). *Xenobiotica* **22**: 1101-1109.

Gibson, G. G. and Skett, P. (1994). *Introduction to Drug Metabolism*. 2nd edition, Blackie Academic and Professional, Glasgow.

Gingell, D., Garrod, D. R. and Palmer, J. F. (1970). In: *Calcium and cellular function*. (A. W. Cuthbert, Ed.), pp.59-64, Macmillan, London.

Ginot, F., Decaux, J.-F., Cognet, M., Berbar, T., Levrat, F., Kahn, A. and Weber, A. (1989). *Eur. J. Biochem.* **180**: 289-294.

Gonzalez, F. J. (1989). *Pharmacol. Rev.* **40**: 243-288.

Gonzalez, F. J. (1990). *Pharmac. Ther.* **45**: 1-38.

Gonzalez, F. J. (1992). *TIPS* **13**: 347-352.

Gonzalez, F. J., Jaiswal, A. K. and Nebert, D. W. (1986). *Cold Spring Harb. Symp. Quant. Biol.* **2**: 879-890.

Gonzalez, F. J. and Kasper, C. B. (1982). *J. Biol. Chem.* **257**: 5962-5968.

Gonzalez, F. J., Kimura, S., Song, B.-J., Pastewka, J., Gelboin, H. V. and Hardwick, J. P. (1986b). *J. Biol. Chem.* **261**: 10667-10672.

Gonzalez, F. J., Liu, S. and Yano, M. (1993). *Pharmacogenetics* **3**: 51-57.

Gonzalez, F. J., Skoda, R., Hardwick, J. P., Song, B. J., Umeno, M., McBride, O. W., Kozak, C., Matsunaga, E., Matsunaga, T., Kimura, S., Park, S. S., Yang, C. S., Nebert, D. W., Gelboin, H. V. and Meyer, U. A. (1988). In: *Microsomes and Drug Oxidations*. (J. O. Miners, D. J. Birkett, R. Drew and M. McManus, Ed.), pp.209-215, Taylor & Francis, London.

Gonzalez, F. J., Tukey, R. H. and Nebert, D. W. (1984). *Mol. Pharmacol.* **26**: 117-121.

Grant, M. H., Melvin, M. A. L., Shaw, P., Melvin, W. T. and Burke, M. D. (1985). *FEBS Lett.* **190**: 99-103.

Green, S. (1992). *Biochem. Pharmacol.* **43**: 393-401.

Guengerich, F. P. (1982). *Rev. Biochem. Toxicol.* **4**: 5-12.

Guengerich, F. P. (1987). In: *Mammalian Cytochrome P-450*. (F. P. Guengerich, Ed.), pp.1-54, CRC Press, Boca Raton, Florida.

Guengerich, F. P. (1990). *CRC Critical Rev. Biochem. Molec. Biol* **25**: 97-153.

Guengerich, F. P. (1991). *J. Biol. Chem.* **266**: 10019-10022.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V. and Kaminsky, L. S. (1982). *Biochemistry* **21**: 6019-6030.

Guillouzo, A. (1986). In: *Research in isolated and cultured hepatocytes.* (A. Guillouzo and C. Guguen-Guiloouzo, Ed.), pp.313-332, John Libbey, London.

Guillouzo, A., Morel, F., Ratanasavanh, D., Chesné, C. and Guguen-Guillouzo, C. (1990). *Toxicology in vitro* **4**: 415-427.

Gunsalus, I. C. and Sligar, S. G. (1977). *Adv. Enzym. Relat. Areas Mol. Biol.* **47**: 1-44.

Hahn, C. N., Hansen, A. J. and May, B. K. (1991). *J. Biol. Chem.* **266**: 17031-17039.

Haniu, M., Iyanagi, T., Miller, P., Lee, T. D. and Shively, J. E. (1986). *Biochemistry* **25**: 7906-7911.

Hardwick, J. P., Gonzalez, F. J. and Kasper, C. B. (1983). *J. Biol. Chem.* **258**: 8081-8085.

Hardwick, J. P., Song, B. J., Huberman, E. and Gonzalez, F. J. (1987). *J Biol Chem* **262**: 801-10.

Hatayama, I., Yamada, Y., Tanaka, K., Ichihara, A. and Sato, K. (1991). *Jpn. J. Cancer Res.* **82**: 807-817.

Haugen, D. A., Coon, M. J. and Nebert, D. W. (1976). *J. Biol. Chem.* **251**: 1817-1827.

He, J. S. and Fulco, A. J. (1991). *J. Biol. Chem.* **266**: 7864-7869.

Henry, E. C. and Gasciewicz, T. A. (1987). *Toxicol. Appl. Pharmacol.* **89**: 165-169.

Hershey, J. W. B. (1991). *Annu. Rev. Biochem.* **60**: 717-755.

Hill, R. G., Simmonds, M. A. and Straughan, D. W. (1972). *Br. J. Pharmacol.* **44**: 807-809.

Hiratsuka, A., Sebata, N., Kawashima, K., Okuda, H., Ogura, K., Watabe, T., Satoh, K., Hatayama, I., Tsuchida, S., Ishikawa, T. and Sato, K. (1990). *J. Biol. Chem.* **265**: 11973-11981.

Hlavica, P. and Kehl, M. (1977). *Biochem. J.* **164**: 487-496.

Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A. and Hankinson, O. (1991). *Science* **252**: 954-958.

Howard, R. B., Lee, J. C. and Pesch, L. A. (1973). *J. Cell Biol.* **57**: 642-658.

Humbert, J. R., Hammond, K. B., Hathaway, W. E., Marcoux, J. G. and O'Brien, D. (1970). *Lancet* **i**: 770-771.

Imagawa, M., Osada, S., Koyama, Y., Suzuki, T., Hirom, P. C., Diccianni, M. B., Morimura, S. and Muramatsu, M. (1991). *Biochem. Biophys. Res. Comm.* **179**: 293-300.

Imagawa, M., Osada, S., Okuda, A. and Muramatsu, M. (1991b). *Nucleic Acids Res.* **19**: 5-10.

Ishigaki, S., Abramovitz, M. and Listowsky, I. (1989). *Arch. Biochem. Biophys.* **273**: 265-272.

Israel, D. I. and Whitlock, J. P., Jr. (1984). *J. Biol. Chem.* **259**: 5400-5402.

Issemann, I. and Green, S. (1990). *Nature* **347**: 645-650.

Iyanagi, T. and Mason, H. S. (1973). *Biochemistry* **12**: 2297-2308.

Jaiswal, A. K., Haaparanta, T., Luc, P. V., Schembri, J. and Adesnik, M. (1990). *Nucleic Acids Res* **18**: 4237-42.

Jaiswal, A. K., Rivkin, E. and Adesnik, M. (1987). *Nucleic Acids Res.* **15**: 6755-6759.

Jakoby, W. B., Ed. (1980). *Enzymatic basis of detoxication.* Academic Press, New York.

Jakoby, W. B., Ketterer, B. and Mannervik, B. (1984). *Biochem. Pharmacol.* **33**: 2539-2540.

Jarnagin, W. R., Debs, R. J., Wang, S.-S. and Bissell, D. M. (1992). *Nucleic Acids Res.* **20**: 4205-4211.

Jat, P. S., Noble, M. D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L. and Kioussis, D. (1991). *Proc. Natl. Acad. Sci.* **88**: 5096-5100.

Jat, P. S. and Sharp, P. A. (1986). *J. Virol.* **59**: 746-750.

Jauregui, H. O., Hayner, N. T., Driscoll, J. L., Williams-Holland, R., Lipsky, M. H. and Galletti, P. M. (1981). *In Vitro* **17**: 1100-1110.

Jenkins, J. R., Rudge, K. and Currie, G. A. (1984). *Nature* **312**: 646-649.

Johansson, I., Ekstrom, G., Scholte, B., Puzycki, D., Jonvall, H. and Ingelmann-Sundberg, M. (1988). *Biochemistry* **27**: 1925-1934.

Johnson, E. F. (1992). *TIPS* **13**: 122-126.

Johnson, E. F., Levitt, D. S., Muller-Eberhard, U. and Thorgeirsson, S. S. (1980). *Cancer Res.* **40**: 4456-4459.

Kaderlik, R. K., Weser, E. and Ziegler, D. M. (1991). *Prog. Pharmacol. Clin. Pharmacol.* **3**: 95-103.

Kano, T., Sakai, M. and Muramatsu, M. (1987). *Cancer Res.* **47**: 5626-5631.

Kappus, H. (1986). *Biochem. Pharmacol.* **35**: 1-6.

Katagiri, M., Murakami, H., Yabusaki, Y., Sugiyama, T., Okamoto, M., Yamano, T. and Ohkawa, H. (1986). *J. Biol. Chem.* **100**: 945-954.

Kawajiri, K., Watanabe, J., Gotoh, O., Tagashira, Y., Sogawa, K. and Fujii-Kuriyama, Y. (1986). *Eur. J. Biochem.* **159**: 219-225.

Khani, S. C., Porter, T. D., Fujita, V. S. and Coon, M. J. (1988). *J. Biol. Chem.* **263**: 7170-7175.

Kimura, S., Hardwick, J. P., Kozak, C. A. and Gonzalez, F. J. (1989). *DNA* **8**: 517-526.

Kimura, T., Kodama, M. and Nagata, C. (1983). *Biochem. Biophys. Res. Commun.* **110**: 640-645.

Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. (1984). *Cancer Res.* **44**: 2698-2703.

Kocarek, T. A., Schuetz, E. G. and Guzelian, P. S. (1990). *Mol. Pharmacol.* **38**: 440-444.

Kocarek, T. A., Schuetz, E. G. and Guzelian, P. S. (1993). *Mol. Pharmacol.* **43**: 328-334.

Koop, D. R., Morgan, E. T., Tarr, G. E. and Coon, M. J. (1982). *J. Biol. Chem.* **257**: 8472-8480.

Kramer, K., Ciaramella, G., Edwards, M., Shephard, E. A. and Phillips, I. R. (1994). In: *Cytochrome P450: Biochemistry,Biophysics and Molecular Biology*. (M. C. Lechner, Ed.), pp.655-658, John Libbey Eurotext, Paris.

Kronbach, T., Fischer, V. and Meyer, U. A. (1988). *Clin. Pharmacol. Ther.* **43**: 630-635.

Kuo, C. T., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E. and Crabtree, G. R. (1992). *Nature* **355**: 458-460.

Labbé, D., Jean, A. and Anderson, A. (1988). *DNA* **7**: 253-260.

Laemmli, U. K. (1970). *Nature* **227**: 680-685.

Lai, H.-C. J., Grove, G. and Tu, C.-P. D. (1987). *Nucleic Acids Res.* **14**: 6101-6105.

Lai, H.-C. J., Li, N.-Q., Weiss, M. J., Reddy, C. C. and Tu, C.-P. D. (1984). *J. Biol. Chem.* **259**: 5536-5541.

Lai, H.-C. J., Qian, B., Grove, G. and Tu, C.-P. D. (1988). *J. Biol. Chem.* **263**: 11389-11394.

Lakso, M., Masaki, R., Noshiro, M. and Negishi, M. (1991). *Eur. J. Biochem.* **195**: 477-486.

Lawton, M. P., Cashman, J. R., Cresteil, T., Dolphin, C. T., Elfarra, A., Heins, R. N., Hodgson, E., Kimura, T., Ozols, J., Phillips, I. R., Philpot, R. M., Poulsen, L. L., Rettie, A. E., Shephard, E. A., Williams, D. E. and Ziegler, D. M. (1994). *Arch. Biochem. Biophys.* **308**: 254-257.

Lawton, M. P., Gasser, R., Tynes, R. E., Hodgson, E. and Philpot, R. M. (1990). *J. Biol. Chem.* **265**: 5855-5861.

Lawton, M. P. and Philpot, R. M. (1993). *Pharmacogenetics* **3**: 40-44.

Lee, M.-Y., Clark, J. E. and Williams, D. E. (1993). *Arch. Biochem. Biophys.* **302**: 332-336.

Leighton, J. K. and Kemper, B. (1984). *J. Biol. Chem.* **259**: 11165-11168.

Lemoin, A., Williams, D. E., Cresteil, T. and Leroux, J. P. (1991). *Mol. Pharmacol.* **40**: 211-217.

Lindberg, R. L. and Negishi, M. (1989). *Nature* **339**: 632-634.

Little, P. F. R. and Jackson, I. J. (1987). In: *DNA Cloning: A practical Approach.* vol. III, (D. M. Glover, Ed.), pp.1-18, IRL Press, Oxford.

Lomri, N., Gu, Q. and Cashman, J. R. (1992). *Biochemistry* **89**: 1685-1689.

Lu, A. Y. H. and Coon, M. J. (1968). *J. Biol. Chem.* **243**: 1331-1332.

Lu, A. Y. H., Junk, K. W. and Coon, M. J. (1969). *J. Biol. Chem.* **244**: 3714-3721.

Lubet, R. A., Mayer, R. T., Cameron, J. W., Nims, R. W., Burke, M. D., Wolff, T. and Guengerich, F. P. (1985). *Arch. Biochem. Biophys.* **238**: 43-48.

Machinist, J. M., Dehner, E. W. and Ziegler, D. M. (1968). *Arch. Biochem. Biophys.* **125**: 858-864.

Mannervik, B. (1985). *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**: 357-417.

Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgernstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Wildersten, M. and Wolf, R. C. (1992). *Biochem. J.* **282**: 305-308.

Mannervik, B. and Danielson, U. H. (1988). *CRC Crit. Rev. Biochem.* **23**: 283-337.

Marie, S. and Cresteil, T. (1989). *Biochimica et Biophysica Acta* **1009**: 221-228.

Masters, B. S. S. and Ziegler, D. M. (1971). *Arch. Biochem. Biphys.* **145**: 358-364.

McBratney, S., Chen, C. Y. and Sarnow, P. (1993). *Curr. Opin. Cell Biol.* **5**: 961-965.

McLellan, L. I., Wolf, C. R. and Hayes, J. D. (1989). *Biochem. J.* **258**: 87-93.

Meredith, M. J. (1988). *Cell Biol. Toxicol.* **4**: 405-425.

Merrick, W. C. (1992). *Microbiol. Rev.* **56**: 291-315.

Meyer, D. J., Coles, B., Pemble, S., Gilmore, K. S., Fraser, G. M. and Ketterer, B. (1991). *Biochem. J.* **274**: 409-414.

Meyer, D. J., Gilmore, K. S., Coles, B., Dalton, K., Hulbert, P. B. and Ketterer, B. (1991b). *Biochem. J.* **274**: 619-623.

Morel, F., Beaune, P. H., Ratanasavanh, D., Flinois, J.-P., Yang, C. S., Guengerich, F. P. and Guillouzo, A. (1990). *Eur. J. Biochem.* **191**: 437-444.

Morgenstern, R., De Pierre, J. W. and Jornvall, H. (1985). *J. Biol. Chem.* **260**: 13976-13981.

Morgenstern, R., Lundqvist, G., Hancock, V. and Depierre, J. W. (1988). *J. Biol. Chem.* **263**: 6671-6675.

Morton, M. R., Bayney, R. M. and Pickett, C. B. (1990). *Arch. Biochem. Biophys.* **277**: 56-62.

Moura Neto, V., Mallat, M., Chneiweiss, H., Premont, J., Gros, F. and Prochiantz, A. (1986). *Dev. Brain Res.* **26**: 11-22.

Muakkassah-Kelly, S. F., Jans, D. A., Lydon, N., Bieri, F., Wacchter, F., Bently, P. and Staubli, W. (1988). *Exp. Cell Res.* **178**: 296-306.

Nakamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. and Ichihara, A. (1983). *Proc. Natl. Acad. Sci.* **80**: 7229-7233.

Nazar-Stewart, V., Motulsky, A. G., Eaton, D. L., White, E., Hornung, S. K., Leng, Z. T., Stapleton, P. and Weiss, N. S. (1993). *Cancer Res.* **53**: 2113-2118.

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. and Waterman, M. R. (1987). *DNA* **6**: 1-11.

Nebert, D. W. and Gonzalez, F. J. (1987). *Ann. Rev. Biochem.* **56**: 945-993.

Nebert, D. W. and Jones, J. E. (1989). *Int. J. Biochem.* **21**: 243-252.

Nebert, D. W., Jones, J. E., Owens, J. and Puga, A. (1988). *Prog Clin Biol Res* **274**: 557-76.

Nebert, D. W. and Nelson, D. R. (1991). *Methods Enzymol* **206**: 3-11.

Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. and Waterman, M. R. (1989). *DNA* **8**: 1-13.

Nebert, D. W., Nelson, D. R., Coon, M. J., Estabrook, R. W., Feyereisen, R., Fujii-kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R. and Waxman, D. J. (1991). *DNA Cell. Biol.* **10**: 1-14.

Negishi, M. and Nebert, D. W. (1981). *J. Biol. Chem.* **256**: 3085-3091.

Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K. and Nebert, D. W. (1993). *DNA Cell. Biol.* **12**: 1-51.

Nelson, D. R. and Strobel, H. W. (1988). *J. Biol. Chem.* **263**: 6038-6050.

Nemunaitis, J., Andrews, D. F., Crittenden, C., Kaushansky, K. and Singer, J. W. (1989). *J. Clin. Invest.* **83**: 593-601.

Nguyen, V. T., Morange, M. and Bensaude, O. (1988). *Anal. Biochem.* **171**: 404-408.

Noshiro, M., Lasko, M., Kawajiri, K. and Negishi, M. (1988). *Biochemistry* **27**: 6434-6443.

Oinonen, T. and Lindros, K. O. (1994). In: *Cytochrome P450: Biochemistry, Biophysics and Molecular Biology*. (M. C. Lecner, Ed.), pp.833-835, European Publishers Ltd., Lisbon, Portugal.

Okey, A. B. (1990). *Pharmacol. Ther.* **45**: 241-298.

Okey, A. B., Roberts, E. A., Harper, P. A. and Denison, M. S. (1986). *Clin. Biochem.* **19**: 132-141.

Okuda, A., Imagawa, M., Maeda, Y., Sakai, M. and Muramatsu, M. (1989). *J. Biol. Chem.* **264**: 16919.

Okuda, A., Sakai, M. and Muramatsu, M. (1987). *J. Biol. Chem.* **262**: 3858-3863.

Olsen, R. W. and Venter, J. C., Ed. (1986). *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties.* Liss, New York.

Omiecinski, C. J. (1986). *Nucleic Acids Res.* **14**: 1525-1539.

Omiecinski, C. J., Hassett, C. and Costa, P. (1990). *Mol. Pharmacol.* **38**: 462-470.

Omura, T. (1979). *Pharmacol. Ther.* **8**: 489-499.

Omura, T. and Sato, R. (1964). *J. Biol. Chem.* **239**: 2370-2378.

Omura, T. and Sato, R. (1964b). *J. Biol. Chem.* **239**: 2379-2385.

Oshino, N. and Sato, R. (1971). *J. Biochem.* **69**: 169-180.

Ozols, J. (1990). *J. Biol. Chem.* **265**: 10289-10299.

Ozols, J. (1991). *Arch. Biochem. Biophys.* **290**: 103-115.

Padgham, C. R., Paine, A. J., Phillips, I. R. and Shephard, E. A. (1992). *Biochem. J.* **285**: 929-932.

Park, B. K. and Breckenridge, A. M. (1981). *Clin. Parmacokinet.* **6**: 1-24.

Parker-Ponder, K., Dunbar, R. P., Wilson, D. R., Darlington, G. J. and Woo, S. (1991). *Human Gene Therapy* **2**: 41-52.

Pasco, D. S., Boyum, K. W., Merchant, S. N., Chalberg, S. C. and Fagan, J. B. (1988). *J. Biol. Chem.* **263**: 8671-8676.

Pasco, D. S. and Fagan, J. B. (1989). *DNA* **8**: 535-541.

Paul, D., Hohne, M., Pinkert, C., Piasecki, A., Ummelmann, E. and Brinster, R. L. (1988). *Exp. Cell Res.* **175**: 354-362.

Paulson, K. E., Darnell, J. E., Jr., Rushmore, T. H. and Pickett, C. B. (1990). *Mol. Cell. Biol.* **10**: 1841-1852.

Pemble, S., Taylor, J. B. and Ketterer, B. (1986). *Biochem. J.* **240**: 885-889.

Petit, C. A., Gardes, M. and Feunteun, J. (1983). *Virology* **127**: 74-82.

Phelps, W. C., Lee, C. L., Munger, K. and Howley, P. M. (1988). *Cell* **53**: 539-547.

Phillips, I. R., Shephard, E. A., Bayney, R. M., Pike, S. F., Rabin, B. R., Heath, R. and Carter, N. (1983). *Biochem J* **212**: 55-64.

Phillips, I. R., Shephard, E. A., Griffith, C., Santisteban, I., Gammon, B. M., Hutter, A., Bell, D. R. and Forrest, L. A. (1987). In: *Microsomes and Drug Oxidations.* (J. Miners, D. J. Birkett, R. Drew and M. McManus, Ed.), pp.89-95, Taylor and Francis, London.

Phillips, I. R., Shephard, E. A., Mitani, F. and Rabin, B. R. (1981). *Biochem. J.* **196**: 839-51.

Pickett, C. B., Telakowski-Hopkins, C. A., Ding, C. J.-F., Argenbright, L. and Lu, A. Y. H. (1984). *J. Biol. Chem.* **259**: 5182-5188.

Pickett, C. P. and Lu, A. Y. H. (1989). *Annu. Rev. Biochem.* **58**: 743-764.

Pike, S. F., Shephard, E. A., Rabin, B. R. and Phillips, I. R. (1985). *Biochem. Pharmacol.* **34**: 2489-2494.

Pitot, J. C., Peraino, C., Moore, P. A. and Potter, V. R. (1964). *Nat. Cancer Inst. Monogr.* **12**: 229-246.

Poland, A. and Knutson, J. C. (1982). *Ann. Rev. Pharmacol. Toxicol.* **22**: 517-554.

Pongratz, I., Mason, G. G. and Poellinger, L. (1992). *J. Biol. Chem.* **267**: 13728-13734.

Porter, T. D., Beck, T. W. and Kasper, C. B. (1990). *Biochemistry* **29**: 9814-9818.

Porter, T. D. and Coon, M. J. (1991). *J. Biol. Chem.* **266**: 13469-13472.

Puga, A. and Nebert, D. W. (1990). *Biochem. Soc. Trans.* **18**: 7-10.

Rampersaud, A. and Walz, F. G., Jr. (1983). *Proc. Natl. Acad. Sci* **80**: 6542-6546.

Ramsden, R., Sommer, K. M. and Omiecinski, C. J. (1993). *J. Biol. Chem.* **268**: 21722-21726.

Rangarajan, P. N. and Padmanaban, G. (1989). *Proc. Natl. Acad. Sci.* **86**: 3963-3967.

Rassoulzadegan, M., Cowie, A., Carr, A., Glaichenhaus, N., Kamen, R. and Cuzin, F. (1982). *Nature* **300**: 713-718.

Reid, L. M. (1990). *Curr. Opin. Cell Biol.* **2**: 121-130.

Remmer, H. and Merker, H. J. (1963). *Science* **142**: 1657-1658.

Reuber, M. D. (1961). *J. Nat. Cancer Inst.* **26**: 891-899.

Reyes, H., Reisz-Porszasz, S. and Hankinson, O. (1992). *Science* **256**: 1193-1195.

Rhoads, R. E. (1991). *Curr. Opin. Cell Biol.* **3**: 1019-1024.

Rippe, R. A., Brenner, D. A. and Leffert, H. L. (1990). *Mol. Cell. Biol.* **10**: 689-695.

Rosenthal, N. (1987). In: *Methods in Enzymology*. 152, (S. L. Berger and A. R. Kimmel, Ed.), pp.704-720, Academic Press, New York.

Rushmore, T. H., King, R. G., Paulson, E. K. and Pickett, C. B. (1990). *Proc. Natl. Acad. Sci.* **87**: 3826-3830.

Ryan, D. E. and Levin, W. (1990). *Pharmacol. Ther.* **45**: 153-239.

Ryan, D. E., Thomas, P. E. and Levin, W. (1982). *Arch. Biochem. Biophys.* **216**: 272-288.

Ryerson, C. C., Ballou, D. P. and Walsh, C. Z. (1982). *Biochemistry* **21**: 2644-2655.

Sabourin, P. J., Smyser, B. P. and Hodgson, E. (1984). *Int. J. Biochem.* **16**: 713-720.

Sakaguchi, M. and Omura, T. (1993). In: *Frontiers in Biotransformation*. vol. 8, (K. Ruckpaul and H. Rein, Ed.), pp.59-73, Akademie-Verlag, Berlin.

Sakai, M., Okuda, A., Hatayama, I., Sato, K., Nishi, S. and Muramatsu, M. (1989). *Cancer Res.* **49**: 5633-5637.

Sakai, M., Okuda, A. and Muramatsu, M. (1988). *Proc. Natl. Acad. Sci.* **85**: 9456-9460.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. (1985). *Proc. Natl. Acad. Sci* **82**: 3964-3968.

Schafer, D. A. and Hultquist, D. E. (1980). *Biochem. Biophys. Res. Commun.* **95**: 381-387.

Schladt, L., Thomas, H., Hartmann, R. and Oesch, F. (1988). *Eur. J. Biochem.* **176**: 715-721.

Schuetz, E. G. and Guzelian, P. S. (1984). *J. Biol. Chem.* **259**: 2007-2012.

Schuetz, E. G., Li, D., Omiecinski, C. J., Muller-Eberhard, U., Kleinman, H. K., Elswick, B. and Guzelian, P. S. (1988). *J. Cell. Physiol.* **134**: 309-323.

Schuetz, E. G., Schuetz, J. D., May, B. and Guzelian, P. S. (1990). *J. Biol. Chem.* **256**: 1188-1192.

Schuetz, E. G., Wrighton, S. A., Safe, S. H. and Guzelian, P. S. (1986). *Biochemistry* **25**: 1124-1133.

Schulte-Hermann, R. (1974). *CRC Crit. Rev. Toxicol.* **3**: 97-158.

Seglen, P. O. (1973). *Exp. Cell Res.* **82**: 391-398.

Seglen, P. O. (1973b). *Exp. Cell Res.* **76**: 25-30.

Seglen, P. O. (1976). *Meth. Cell Biol.* **18**: 29-83.

Seidegard, J., Guthenberg, C., Piro, R. W. and Mannervik, B. (1987). *Biochem. J.* **246**: 783-788.

Seidegard, J., Vorachek, W. R., Pero, R. W. and Pearson, W. R. (1988). *Proc. Natl. Acad. Sci.* **85**: 7293-7299.

Sexton, D. J., Dimmock, J. R. and Mutus, B. (1993). *Cell Biol.* **71**: 98-101.

Shaw, G. C. and Fulco, A. J. (1992). *J. Biol. Chem.* **267**: 5515-5526.

Shaw, P. M., Adesnik, M., Weiss, M. C. and Corcos, L. (1993). *Mol. Pharmacol.* **44**: 775-783.

Shaw, W. V. (1975). In: *Methods in Enzymology*. 43, pp.737-755, Academic Press, New York.

Shelley, E. D. and Shelley, W. D. (1984). *J. Am. Med. Assoc.* **251**: 253-255.

Shephard, E. A., Dolphin, C. T., Fox, M. F., Povey, S., Smith, R. and Phillips, I. R. (1993). *Genomics* **16**: 85-89.

Shephard, E. A., Forrest, L. A., Shervington, A., Fernandez, L., Ciaramella, G. and Phillips, I. R. (1994). *DNA Cell. Biol.* **13**: 793-798.

Shephard, E. A., Phillips, I. R., Bayney, R. M., Pike, S. F. and Rabin, B. R. (1983). *Biochem. J.* **211**: 333-40.

Shervington, A., Shephard, E. A. and Phillips, I. R. (1994). In: *Cytochrome P450: Biochemistry, Biophysics and Molecular Biology*. (M. C. Lechner, Ed.), pp.137-140, European Publishers Ltd., Paris.

Sidhu, J. S., Farin, F. M. and Omiecinski, C. J. (1993). *Arch. Biochem. Biophys.* **391**: 103-113.

Sigel, E., Baur, R., Trube, G., Mohler, H. and Malherbe, P. (1990). *Neuron* **5**: 703-711.

Simmons, D. L., McQuiddy, P. and Kaspar, C. B. (1987). *J. Biol. Chem.* **262**: 326-332.

Sinclair, J. F., McCaffrey, J., Sinclair, P. R., Bement, W. J., Lambrecht, L. K., Wood, S. G., Smith, E. L., Schenkman, J. B., Guzelian, P. S., Park, S. S. and Gelboin, H. V. (1991). *Arch. Biochem. Biophys.* **284**: 360-365.

Sinclair, P. R., Bement, W. J., Haugen, S. A., Sinclair, J. F. and Guzelian, P. S. (1990). *Cancer Res.* **50**: 5219-5224.

Sirica, A. E. and Pitot, H. C. (1980). *Pharmacol. Rev.* **31**: 205-228.

Skett, P. (1987). In: *Progress in drug metabolism*. vol. 10, (J. W. Bridges, L. F. Chasseaud and G. G. Gibson, Ed.), pp.85-140, Taylor & Francis, London.

Skett, P. (1988). *Pharmacol. Therap.* **38**: 269-304.

Sladek, F. M. and Darnell, J. E. (1992). *Curr. Opin. Genes Dev.* **2**: 256-259.

Smyser, B. P. and Hodgson, E. (1985). *Biochem. Pharmacol.* **34**: 1145-1150.

Song, B. J., Gelboin, H. V., Park, S.-S., Yang, S. C. and Gonzalez, F. J. (1986). *J. Biol. Chem.* **261**: 16689-16697.

Strittmater, P., Fleming, P., Connors, M. and Corcoran, D. (1978). In: *Methods in Enzymology*. vol. LII, (S. Fleischer and L. Packer, Ed.), pp.97-101, Acadenic Press, New York.

Strom, S. C., Jirtle, R. L., Jones, R. S., Novicki, D. L., Rosemberg, M. R., Novotny, A., Irons, G., McLain, J. R. and Michalopoulos, G. (1982). *J. Nat. Cancer Inst.* **68**: 771-778.

Stupans, I., Ikeda, T., Kessler, D. J. and Nebert, D. W. (1984). *DNA Cell. Biol.* **3**: 129-137.

Sugioka, Y., Fujii-Kuriyama, Y., Kitagawa, T. and Muramatsu, M. (1985). *Cancer Res.* **45**: 365-371.

Suwa, Y., Mizukami, Y., Sogawa, K. and Fujii-Kuriyama, Y. (1985). *J. Biol. Chem.* **260**: 7980-7984.

Swanson, H. I. and Bradfield, C. A. (1993). *Pharmacogenetics* **3**: 213-230.

Tan, K. H., Meyer, D. J., Gillies, N. and Ketterer, B. (1988). *Biochem. J.* **254**: 841-845.

Taylor, J. B., Craig, R. K., Beale, D. and Ketterer, B. (1984). *Biochem. J.* **219**: 223-231.

Taylor, J. B., Oliver, J., Sherrington, R. and Pemble, S. E. (1991). *Biochem. J.* **274**: 587-600.

Tee, L. B. G., Gilmore, K. S., Meyer, E. J., Ketterer, B., Vandenberghe, Y. and Yeoh, G. C. T. (1992). *Biochem. J.* **282**: 209-218.

Telakowski-Hopkins, C. A., King, R. G. and Pickett, C. B. (1988). *Proc. Natl. Acad. Sci.* **85**: 1000-1005.

Telakowski-Hopkins, C. A., Rothkopf, G. S. and Pickett, C. B. (1986). *Proc. Natl. Acad. Sci.* **83**: 9393-9398.

Tephly, T. R. and Burchell, B. (1990). *Trends Pharmacol. Sci.* **11**: 276-279.

Thomas, P. E., Reik, D. E., Ryan, D. E. and Levin, W. (1981). *J. Biol. Chem.* **256**: 1044-1052.

Tierney, B. and Bresnik, E. (1981). *Arch. Biochem. Biophys.* **210**: 729-739.

Traber, P. G., Maganto, P., Wojcik, E., Keren, D. and Gumucio, J. J. (1989). *J. Biol. Chem.* **264**: 10292-10298.

Traber, P. G., Wang, W., McDonnell, M. and Gumucio, J. J. (1990). *Mol. Pharmacol.* **37**: 810-819.

Tynes, R. E. and Hodgson, E. (1985). *Arch. Biochem. Biophys.* **240**: 77-93.

Ueno, T. and Gonzalez, F. J. (1990). *Mol. Cell Biol.* **10**: 4495-4505.

Umeno, M., McBride, O. W., Yang, C. S., Gelboin, H. V. and Gonzalez, F. J. (1988). *Biochemistry* **26**: 9006-9013.

Umeno, M., Song, B. J., Kozak, C., Gelboin, H. V. and Gonzalez, F. J. (1988b). *J. Biol. Chem.* **263**: 4956-4962.

Urenjak, J., Linder, D. and Lumper, L. (1987). *J. Chromat.* **397**: 123-136.

Van Bladeren, P. J., Ploemen, J. and Van Ommen, B. (1993). In: *Developments and ethical considerations in toxicology.* (M. I. Weitzner, Ed.), pp.69-85, Royal Society of Chemistry, Cambridge.

Vandenberghe, Y., Glaise, D., Meyer, D. J., Guillouzo, A. and Ketterer, B. (1988). *Biochem. Pharmacol.* **37**: 2482-2485.

Vandenberghe, Y., Morel, F., Foriers, A., Ketterer, B., Vercruysee, A., Guillouzo, A. and Rogiers, V. (1989). *FEBS Lett.* **251**: 59-64.

Vandenberghe, Y., Morel, F., Pemble, S., Taylor, J. B., Rogiers, V., Ratanasavanh, D., Vercruysse, A., Ketterer, B. and Guillouzo, A. (1990). *Mol. Pharmacol.* **37**: 372-376.

Vandenberghe, Y., Tee, L., Rogiers, V. and Yeoh, G. (1992). *FEBS Lett.* **313**: 155-159.

Vermillon, J. L. and Coon, M. J. (1974). *Biochem. Biophys. Res. Commun.* **60**: 1315-1322.

Vorachek, W. R., Pearson, W. R. and Rule, G. S. (1991). *Proc. Natl. Acad. Sci.* **88**: 4443-4449.

Vos, R. M. and Van Bladeren, P. J. (1990). *Chem. Biol. Interact.* **75**: 241-265.

Watkins, P. B., Wrighton, S. A., Schuetz, E. G., Maurel, P. and Guzelian, P. S. (1986). *J. Biol. Chem.* **261**: 6264-6271.

Watson, A. J., Weir-Brown, K. I., Bannister, R. M., Chu, F. F., Reisz-Porszasz, S., Fujii-Kuriyama, Y., Sogawa, K. and Hankinson, O. (1992). *Mol. Cell. Biol.* **12**: 2115-2123.

Waxman, D. J. (1988). *Biochem. Pharmacol.* **37**: 71-84.

Waxman, D. J. and Azaroff, L. (1992). *Biochem. J.* **281**: 577-592.

Waxman, D. J., Dannan, G. A. and Guengerich, F. P. (1985). *Biochemistry* **24**: 4409-4417.

Waxman, D. J., Ko, A. and Walsh, C. (1983). *J. Biol. Chem.* **258**: 11937-11947.

Waxman, D. J., Morissey, J. J., Naik, S. and Jauregui, H. O. (1990). *Biochem J.* **271**: 113-119.

Waxman, D. J. and Walsh, C. (1982). *J. Biol. Chem.* **257**: 10446-10457.

Wen, L. P., Ruettinger, R. R. and Fulco, A. J. (1989). *J. Biol. Chem.* **264**: 10996-11003.

White, R. E. (1994). In: *Cytochrome P450: Biochemistry, Biophysics and Molecular Biology*. (M. C. Lechner, Ed.), pp.333-340, John Libbey Eurotext, Paris.

Yabusaki, Y., Murakami, H. and Ohkawa, H. (1988). *J. Biol. Chem.* **103**: 1004-1010.

Yamada, H., Fujisaki, H., Kaneko, H., Ishii, Y., Hamaguchi, T. and Oguri, K. (1993). *Biochem. Pharmacol.* **45**: 1783-1789.

Yamano, S., Aoyama, T., McBride, O. W., Hardwick, J. P., Gelboin, H. V. and Gonzalez, F. J. (1989). *Molec. Pharmac.* **35**: 83-88.

Yamazoe, Y., Shimada, M. and Kato, R. (1987). *J. Biol. Chem.* **262**: 7423-7428.

Yanai, N., Suzuki, M. and Obinata, M. (1991). *Exp. Cell Res.* **197**: 50-56.

Yang, C. S., Yoo, J.-S. H., Ishizaki, H. and Hong, Y. (1990). *Drug Metabolism Rev.* **22**: 147-159.

Zaret, K. S., DiPersio, C. M., Jackson, D. A., Montigny, W. J. and Weinstat, D. L. (1988). *Proc. Natl. Acad. Sci.* **85**: 9076-9080.

Ziegler, D. M. (1980). In: *Enzymatic basis of detoxification.* 1, (W. B. Jakoby, Ed.), pp.201-207, Academic Press, New York.

Ziegler, D. M. (1988). *Drug Metab. Rev.* **19**: 1-32.

Ziegler, D. M. (1991). In: *N-oxidation of drugs: biochemistry, pharmacology, toxicology.* (P. Hlavic and L. A. Damani, Ed.), pp.59-68, Chapman & Hall, London.

Ziegler, D. M. (1993). *Annu. Rev. Pharmacol. Toxicol* **33**: 179-199.

Ziegler, D. M. and Mitchell, C. H. (1972). *Arch. Biochem. Biophys.* **150**: 116-125.

Zinn, K., di Maio, D. and Maniatis, T. (1984). *Cell* **34**: 865-879.